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(71) Applicant (for all designated States except US): SMITHKLINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, Philadelphia, PA 19103 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): ERICKSON-MILLER, Connie, L. [US/US]; 608 Brainerd Place, Exton, PA 19341 (US). HOLMES, Stephen, D. [GB/GB]; New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). TAYLOR, Alexander, H. [US/US]; 522 Westfield Drive, Exton, PA 19341 (US). YOUNG, Peter, R. [US/US]; 109 Hayman Place, Wilmington, DE 19803 (US).			
(74) Agents: BAUMEISTER, Kirk et al.; Smithkline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).			
(54) Title: ERYTHROPOIETIN RECEPTOR ANTIBODIES			
(57) Abstract Erythropoietin receptor agonist and antagonist antibodies and their use in enhancing erythropoiesis are disclosed.			

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ERYTHROPOIETIN RECEPTOR ANTIBODIES

Field of the Invention

This invention relates to agonist monoclonal antibodies (mAb) that bind to the erythropoietin receptor (EpoR) and to the use of such antibodies for therapeutic purposes. This invention also relates to antagonist monoclonal antibodies (mAb) that bind to the erythropoietin receptor (EpoR) and to the use of such antibodies for therapeutic purposes.

Background of the Invention

Erythropoietin (Epo) is the naturally occurring hematopoietic growth factor required for the production of mature red blood cells. Epo has a molecular mass of 18.4 kD excluding carbohydrate, and when naturally glycosylated is 35 kD (Roberts, D. and Smith, D.J., *J. Mol. Endocrinology* 12, 131, 1994). The protein is encoded by only one gene (Youssoufian, H., Zon, L.I., Orkin, S.H., D'Andrea, A.D. & Lodish, H.F. *Mol. Cell. Biol.* 10, 3675-3682 (1990), Maouche, L., *et al. Blood* 78, 2557-2563 (1991)). This growth factor stimulates the proliferation of early and late erythroid specific progenitor cells as well as the hemoglobinization of proerythroblasts and their differentiation into mature red blood cells.

Recombinant human Epo (rEpo) has an established market and is routinely used in the care of patients with renal failure, where kidney damage results in anemia due to insufficient production of Epo (Foa, P. *Acta Haematol.* 86, 162-168 (1991)). Furthermore, Epo has also been shown to be useful in specific clinical settings, such as autologous blood transfusion prior to elective surgery, prevention and/or treatment of anemia induced by cytoreductive drugs, and for the treatment of anemia patients receiving zidovudine for HIV infection (Ascensao, J.A., Bilgrami, S. & Zanjani, E.D. *Am. J. Pediat. Hematol.* 13, 376-387 (1991)). Therapy with rEpo is remarkably well tolerated by most patients with few, if any, major adverse reactions reported. Despite the success of rEpo in the clinic, its full potential has not been realized due to limitations imposed by the short half life of rEpo that require frequent dosing and the high cost of treatment. The current recommended dosing is 3x a week delivered subcutaneously.

Epo is a member of a family of structurally and genetically related ligands, which include IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, LIF, G-CSF, GM-CSF, M-CSF, Epo, growth hormone and PRL (see Young, P.R. *Curr. Opin. Biotech.* 3, 408, (1992) for review). The structures of several of the ligands have been determined by X-ray crystallography and/or NMR, and all have a basic core structure of a four α -

helical bundle with an up-up-down-down connectivity. A similar fold is predicted for other members of the family based on modeling and gene structure.

Epo acts through a cell surface receptor which belongs to the hematopoietic cytokine receptor family. EpoR has been cloned from mouse and human and exists in 5 both membrane-bound and secreted forms (D'Andrea, A.D., Lodish, H.F. & Wong, G.G. *Cell* 57, 277-285 (1989), Jones, S.S., D'Andrea, A.D., Haines, L.L. & Wong, G.G. *Blood* 76, 31-35 (1990), Nakamura, Y., Komatsu, N. & Nakauichi, H. *Science* 257, 1138-1141 (1992) and Todokoro, K., Kuramochi, T., Nagasawa, T., Abe, T. & Ikawa, Y. *Gene* 106, 283-284 (1991)). The extracellular domain of the receptors contains the 10 "hematopoietic motif" which consists of two 100 amino acid long fibronectin-like domains and a conserved WSXWS sequence motif, while the intracellular domains contain several conserved regions but do not encode an endogenous kinase activity. Examination of the growth hormone ligand-receptor complex structure (De Vos, A.M., Ultsch, M. & Kossiakoff, A.A. *Science* 255, 306, (1992)) and extensive mutagenesis of 15 the ligands (reviewed in Young, P.R., *supra*,) suggests that, in general, the interaction between receptor and ligand is similar for all members of the hematopoietic cytokine family, with the loops of the two fibronectin domains of each receptor subunit interacting with the amino and carboxy-terminal α -helices.

20 All ligands in this family stimulate biological activity by causing the aggregation of single or multiple receptor subunits in target cells. In the case of Epo, the critical event appears to be the dimerization of a single receptor subunit. Mutant cloned receptors which lead to constitutively active, ligand-independent growth in transfected cell lines, are constitutively dimeric (Watowich, S.S., *et al. Proc. Natl. Acad. Sci. USA* 89, 2140, (1992)). Furthermore, *in vitro* studies of complex formation 25 between Epo and the extracellular domain of EpoR suggest a 1:2 ligand:receptor interaction (Harris, K.W., Mitchell, R.A. & Winkelmann, J.C. *J. Biol. Chem.* 267, 15205, (1992), Philo, J.S., Aoki, K.H., Arakawa, T., Narhi, L.O. & Wen, J. *Biochemistry* 35, 1681, (1996)). More recently a peptide with Epo mimetic activity 30 was shown to dimerize the receptor (Wrighton, N.C. *et al., Science* 273, 458-463 (1996)).

35 The interaction of Epo with its receptor initiates a chain of events involving tyrosine and serine-threonine protein kinases which culminate in changes in the pattern of cellular gene expression, proliferation and differentiation. While there have been many advances in the understanding of the signal transduction pathways following Epo binding to its receptor (for a review see: Ihle, J. N. *Nature* 377, 591-594 (1995)), it is still not clear how progenitor cells decide between proliferation and differentiation.

The finding that dimerization of the receptor is a key step in the stimulation of mitogenesis by Epo suggests another approach to novel Epo-like agonists. In at least three examples of other receptors where homodimerization is induced by receptor binding, monoclonal antibodies have been developed which also had agonist properties.

5 These include monoclonal antibodies to EGF, TNF and growth hormone receptors (Schreiber, A.B., Libermann, T.A., Lax, I., Yarden, Y. & Schlessinger, J. *J. Biol. Chem.* 258, 846-853 (1983), Defize, L.H.K., Moolenaar, W.H., van der Saag, P.T. & de Laat, S.W. *EMBO J.* 5, 1187-1192 (1986), Engelmann, H., *et al.* *J. Biol. Chem.* 265, 14497-14504 (1990), Fuh, G., *et al.* *Science* 256, 1677-1680 (1992)). In all three cases, the

10 monoclonal antibody, by virtue of its two antigen recognition sites, was able to bring together two receptors and activate them. Fab fragments made from these mAbs were inactive. In some cases, the apparent affinity of the antibody for receptor was comparable to that of the ligand (e.g., growth hormone, Fuh, G., *et al.* *Science* 256, 1677-1680 (1992)). More recently, there were reports of monoclonal antibodies raised

15 to the Epo receptor that have Epo-like activity (Schneider, H. *et al.*, *Blood* 89, 473-482 (1997) and Elliot, E. *Et al.*, *J. Biol. Chem.* 271, 24691-24697 (1996)). However, these reports indicated that the frequency of obtaining agonist monoclonal antibodies to the Epo receptor was very low, and their potency was low and hence unsuitable for use therapeutically.

20 Clearly, there is a need to develop high affinity, potent agonist antibodies to the EpoR which will have sufficient activity to work *in vivo* at therapeutically acceptable concentrations.

There are available well known methods for humanization of non-human mAbs that result in less immunogenic antibodies for human therapy, yet retain full binding 25 avidity. These methods can be applied to receptor agonist mAbs whose mode of action is the dimerization of receptors in a manner that mimics the action of the natural receptor ligand.

30 A humanized agonist mAb with equal or better affinity than rEpo for its receptor and an appropriate Fc region would be expected to have a longer *in vivo* half-life. This would be expected to produce an Epo-like protein with a lower frequency of dosing compared to rEpo, which is presently given three times a week by subcutaneous injection.

Summary of the Invention

35 One aspect of the present invention is a method for enhancing erythropoiesis in a animal comprising administering an effective dose of an erythropoietin receptor agonist antibody having the identifying characteristics of monoclonal antibody 3G9; 1-

0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k, 1-0 IgG1,6-0k; or 1-0 IgG4PE,6-0k.

Another aspect of the invention is an EpoR agonist antibody having the identifying characteristics of monoclonal antibody 3G9; 1-0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k, 1-0 IgG1,6-0k; or 1-0 IgG4PE,6-0k.

Another aspect of the invention is a hybridoma having the identifying characteristics of cell line 3G9.

Yet another aspect of the invention is an EpoR agonist antibody comprising a 10 V_H amino acid sequence as set forth in SEQ ID NO: 2 and a V_L amino acid sequence as set forth in SEQ ID NO: 4.

Yet another aspect of the invention is an EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 16.

15 Yet another aspect of the invention is an EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 14 and a V_L amino acid sequence as set forth in SEQ ID NO: 16.

Yet another aspect of the invention is an EpoR agonist antibody comprising a 20 V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 18.

Yet another aspect of the invention is an EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 20.

25 Yet another aspect of the invention is an EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 22.

Yet another aspect of the invention is an EpoR agonist antibody comprising a 30 V_H amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 12 or 14.

Yet another aspect of the invention is an EpoR agonist antibody comprising a V_L amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 16, 18, 20 or 22.

35 Yet another aspect of the invention is an immunoglobulin heavy chain complementarity determining region, the amino acid sequence of which is selected from the group consisting of SEQ ID NOs: 5, 6 and 7.

Yet another aspect of the invention is an immunoglobulin light chain complementarity determining region, the amino acid sequence of which is selected from the group consisting of SEQ ID NOs: 8, 9 and 10.

5 Yet another aspect of the invention is an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 2, 4, 12, 14, 16, 18, 20 or 22 and functional fragments or analogs therof.

Brief Description of the Drawings

10 Figure 1 is a graph of experimental results demonstrating the activity of monoclonal antibodies 3G9 and 3B3 in the UT7-Epo cell proliferation assay.

Figure 2 is a graph of experimental results demonstrating the activity of monoclonal antibodies 3G9 and 3B3 in the human bone marrow CFU-E assay.

15 Figure 3 is a graph of experimental results demonstrating the activity of humanized monoclonal antibodies 1-0 IgG4PE, REIk, S14 IgG4PE, 1-0k and 3G9 in the human bone marrow CFU-E assay.

Figure 4 is a graph of experimental results demonstrating the activity of humanized monoclonal antibodies 1-0 IgG4PE, REIk and 3G9 in the primate bone marrow CFU-E assay.

20 Figure 5 is a graph of experimental results demonstrating the activity of Epo and the monoclonal antibodies 3G9 and 3B3 in the rabbit bone marrow CFU-E assay.

Figure 6 is a graph of experimental results demonstrating JAK2 activation of UT7-Epo cells by Epo and the monoclonal antibody 3G9.

Detailed Description of the Invention

25 All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

As used herein, the term "enhancing erythropoiesis" and "erythropoietic" means increasing the production of erythrocytes as well as increasing the production of precursors and components of erythrocytes.

30 As used herein, the term "decreasing erythropoiesis" and derivatives thereof means decreasing the production of erythrocytes as well as decreasing the production of precursors and components of erythrocytes.

As used herein, the term "agonist activity" refers to the activity of an antibody that binds to human EpoR and enhances erythropoiesis.

35 As used herein, the term "antagonist activity" refers to the activity of an antibody that binds to human EpoR and decreases erythropoiesis.

As used herein, the term "treating" and derivatives thereof means prophylactic or therapeutic therapy.

The present invention provides a variety of antibodies, including altered antibodies and fragments thereof directed against EpoR, which are characterized by 5 agonist activity (and by antagonist activity). Exemplary anti-EpoR agonist antibodies are the murine monoclonal antibody 3G9 and humanized derivatives 1-0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k; 1-0 IgG1,6-0k; and 1-0 IgG4PE,6-0k.

"Antibodies" refers to immunoglobulins which can be prepared by conventional 10 hybridoma techniques, phage display combinatorial libraries, immunoglobulin chain shuffling and humanization techniques. Also included are fully human monoclonal antibodies. As used herein, "antibody" also includes "altered antibody" which refers to a protein encoded by an altered immunoglobulin coding region, which may be obtained by expression in a selected host cell. Such altered antibodies are engineered antibodies 15 (e.g., chimeric or humanized antibodies) or antibody fragments lacking all or part of an immunoglobulin constant region, e.g., Fv, Fab, Fab' or F(ab')₂ and the like. These antibody products are useful in therapeutic and pharmaceutical compositions for treating anemias, cytopenias, acute renal failure and other conditions with depressed erythrocyte production.

20 "Altered immunoglobulin coding region" refers to a nucleic acid sequence encoding an altered antibody of the invention. When the altered antibody is a complementarity determining region-grafted (CDR-grafted) or humanized antibody, the sequences that encode the CDRs from a non-human immunoglobulin are inserted into a first immunoglobulin partner comprising human variable framework sequences.

25 Optionally, the first immunoglobulin partner is operatively linked to a second immunoglobulin partner.

30 "First immunoglobulin partner" refers to a nucleic acid sequence encoding a human framework or human immunoglobulin variable region in which the native (or naturally-occurring) CDR-encoding regions are replaced by the CDR-encoding regions of a donor antibody. The human variable region can be an immunoglobulin heavy chain, a light chain (or both chains), an analog or functional fragments thereof. Such CDR regions, located within the variable region of antibodies (immunoglobulins) can be determined by known methods in the art. For example Kabat *et al.* in "Sequences of Proteins of Immunological Interest", 4th Ed., U.S. Department of Health and Human 35 Services, National Institutes of Health (1987) disclose rules for locating CDRs. In addition, computer programs are known which are useful for identifying CDR regions/structures.

"Second immunoglobulin partner" refers to another nucleotide sequence encoding a protein or peptide to which the first immunoglobulin partner is fused in frame or by means of an optional conventional linker sequence (*i.e.*, operatively linked). Preferably, it is an immunoglobulin gene. The second immunoglobulin 5 partner may include a nucleic acid sequence encoding the entire constant region for the same (*i.e.*, homologous, where the first and second altered antibodies are derived from the same source) or an additional (*i.e.*, heterologous) antibody of interest. It may be an immunoglobulin heavy chain or light chain (or both chains as part of a single polypeptide). The second immunoglobulin partner is not limited to a particular 10 immunoglobulin class or isotype. In addition, the second immunoglobulin partner may comprise part of an immunoglobulin constant region, such as found in a Fab, or F(ab)₂ (*i.e.*, a discrete part of an appropriate human constant region or framework region). Such second immunoglobulin partner may also comprise a sequence encoding an integral membrane protein exposed on the outer surface of a host cell, *e.g.*, as part of a 15 phage display library, or a sequence encoding a protein for analytical or diagnostic detection, *e.g.*, horseradish peroxidase, β -galactosidase, etc.

The terms Fv, Fc, Fd, Fab, Fab' or F(ab')₂ are used with their standard meanings. See, *e.g.*, Harlow *et al.* in "Antibodies A Laboratory Manual", Cold Spring Harbor Laboratory, (1988).

20 As used herein, an "engineered antibody" describes a type of altered antibody, *i.e.*, a full-length synthetic antibody (*e.g.*, a chimeric or humanized antibody as opposed to an antibody fragment) in which a portion of the light and/or heavy chain variable domains of a selected acceptor antibody are replaced by analogous parts from one or more donor antibodies which have specificity for the selected epitope. For example, 25 such molecules may include antibodies characterized by a humanized heavy chain associated with an unmodified light chain (or chimeric light chain), or vice versa. Engineered antibodies may also be characterized by alteration of the nucleic acid sequences encoding the acceptor antibody light and/or heavy variable domain framework regions in order to retain donor antibody binding specificity. These 30 antibodies can comprise replacement of one or more CDRs (preferably all) from the acceptor antibody with CDRs from a donor antibody described herein.

A "chimeric antibody" refers to a type of engineered antibody which contains a naturally-occurring variable region (light chain and heavy chains) derived from a donor antibody in association with light and heavy chain constant regions derived from an 35 acceptor antibody.

A "humanized antibody" refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining

immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. In addition, framework support residues may be altered to preserve binding affinity. See, e.g., Queen *et al.*, *Proc. Natl Acad Sci USA*, **86**, 10029-10032 (1989), Hodgson *et al.*, *Bio/Technology*, **9**, 421 (1991). Furthermore, as described 5 herein, additional residues may be altered to preserve the agonist activity of the donor antibody.

The term "donor antibody" refers to a monoclonal or recombinant antibody which contributes the nucleic acid sequences of its variable regions, CDRs or other functional fragments or analogs thereof to a first immunoglobulin partner, so as to 10 provide the altered immunoglobulin coding region and resulting expressed altered antibody with the antigenic specificity and neutralizing activity characteristic of the donor antibody. One donor antibody suitable for use in this invention is a murine agonist monoclonal antibody designated as 3G9.

The term "acceptor antibody" refers to monoclonal or recombinant antibodies 15 heterologous to the donor antibody, which contributes all, or a portion, of the nucleic acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions or V region subfamily consensus sequences to the first immunoglobulin partner. Preferably, a human antibody is the acceptor antibody.

20 "CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, e.g., Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987). There are three heavy chain and three light chain CDRs or CDR regions 25 in the variable portion of an immunoglobulin. Thus, "CDRs" as used herein refers to all three heavy chain CDRs, or all three light chain CDRs or both all heavy and all light chain CDRs, if appropriate.

CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor 30 antibody variable heavy and light chain sequences, and include analogs of the naturally occurring CDRs, which analogs share or retain the same antigen binding specificity and/or agonist ability as the donor antibody from which they were derived, yet may exhibit increased affinity for the antigen. An exemplary process for obtaining analogs is affinity maturation by means of phage display technology as reviewed by 35 Hoogenboom, *Trends in Biotechnology* **15**, 62-70 (1997); Barbas *et al.*, *Trends in Biotechnology* **14**, 230-234 (1996); and Winter *et al.*, *Ann. Rev. Immunol.* **12**, 433-455 (1994) and described by Irving *et al.*, *Immunotechnology* **2**, 127-143 (1996).

By "sharing the antigen binding specificity or agonist ability" is meant, for example, that although mAb 3G9 may be characterized by a certain level of agonist activity, a CDR encoded by a nucleic acid sequence of 3G9 in an appropriate structural environment may have a lower or higher activity. It is expected that CDRs of 3G9 in 5 such environments will nevertheless recognize the same epitope(s) as 3G9.

A "functional fragment" is a partial heavy or light chain variable sequence (e.g., minor deletions at the amino or carboxy terminus of the immunoglobulin variable region) which retains the same antigen binding specificity and/or agonist ability as the antibody from which the fragment was derived.

10 An "analog" is an amino acid sequence modified by at least one amino acid, wherein said modification can be chemical or a substitution or a rearrangement of a few amino acids (i.e., no more than 10) and corresponding nucleic acid sequences, which modification permits the amino acid sequence to retain the biological characteristics, e.g., antigen specificity and high affinity, of the unmodified sequence. Exemplary 15 nucleic acid analogs include silent mutations which can be constructed, via substitutions, to create certain endonuclease restriction sites within or surrounding CDR-encoding regions.

20 Analogs may also arise as allelic variations. An "allelic variation or modification" is an alteration in the nucleic acid sequence encoding the amino acid or peptide sequences of the invention. Such variations or modifications may be due to degeneracy in the genetic code or may be deliberately engineered to provide desired characteristics. These variations or modifications may or may not result in alterations 25 in any encoded amino acid sequence.

The term "effector agents" refers to non-protein carrier molecules to which the 25 altered antibodies, and/or natural or synthetic light or heavy chains of the donor antibody or other fragments of the donor antibody may be associated by conventional means. Such non-protein carriers can include conventional carriers used in the diagnostic field, e.g., polystyrene or other plastic beads, polysaccharides, e.g., as used in the BIACore (Pharmacia) system, or other non-protein substances useful in the 30 medical field and safe for administration to humans and animals. Other effector agents may include a macrocycle, for chelating a heavy metal atom or radioisotopes. Such effector agents may also be useful to increase the half-life of the altered antibodies, e.g., polyethylene glycol.

35 For use in constructing the antibodies, altered antibodies and fragments of this invention, a non-human species such as bovine, ovine, monkey, chicken, rodent (e.g., murine and rat) may be employed to generate a desirable immunoglobulin upon presentation with human EpoR or a peptide epitope therefrom. Conventional

hybridoma techniques are employed to provide a hybridoma cell line secreting a non-human mAb to the EpoR. Such hybridomas are then screened for binding and agonist activity as described in the Examples section. Alternatively, fully human mAbs can be generated by techniques known to those skilled in the art and used in this invention.

5 An exemplary agonist mAb of the present invention is mAb 3G9, a murine antibody which can be used for the development of a chimeric or humanized molecule. The 3G9 mAb is characterized by agonist activity on erythrocyte production as measured by the CFU-E assay and is produced by the hybridoma cell line 3G9. Other exemplary agonist mAbs are disclosed in U.S. Patent Application Serial No.

10 08/960,733.

The present invention also includes the use of Fab fragments or F(ab')₂ fragments derived from mAbs directed against human EpoR as bivalent fragments. These fragments are useful as agents having agonist activity at the human EpoR. A Fab fragment contains the entire light chain and amino terminal portion of the heavy chain.

15 An F(ab')₂ fragment is the fragment formed by two Fab fragments bound by disulfide bonds. The mAbs 3G9 and other similar high affinity antibodies provide sources of Fab fragments and F(ab')₂ fragments which can be obtained by conventional means, e.g., cleavage of the mAb with the appropriate proteolytic enzymes, papain and/or pepsin, or by recombinant methods. These Fab and F(ab')₂ fragments are useful

20 themselves as therapeutic, prophylactic or diagnostic agents, and as donors of sequences including the variable regions and CDR sequences useful in the formation of recombinant or humanized antibodies as described herein.

The Fab and F(ab')₂ fragments can be constructed via a combinatorial phage library (see, e.g., Winter *et al.*, *Ann. Rev. Immunol.*, 12:433-455 (1994)) or via

25 immunoglobulin chain shuffling (see, e.g., Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), wherein the Fd or v_H immunoglobulin from a selected antibody (e.g., 3G9) is allowed to associate with a repertoire of light chain immunoglobulins, v_L (or v_K), to form novel Fabs. Conversely, the light chain immunoglobulin from a selected antibody may be allowed to associate with a repertoire of heavy chain immunoglobulins, v_H (or

30 Fd), to form novel Fabs. EpoR agonist Fabs can be obtained by allowing the Fd of mAb 3G9 to associate with a repertoire of light chain immunoglobulins. Hence, one is able to recover neutralizing Fabs with unique sequences (nucleotide and amino acid) from the chain shuffling technique.

The mAb 3G9 or other antibodies described above may contribute sequences,

35 such as variable heavy and/or light chain peptide sequences, framework sequences, CDR sequences, functional fragments, and analogs thereof, and the nucleic acid

sequences encoding them, useful in designing and obtaining various altered antibodies which are characterized by the antigen binding specificity of the donor antibody.

The nucleic acid sequences of this invention, or fragments thereof, encoding the variable light chain and heavy chain peptide sequences are also useful for mutagenic 5 introduction of specific changes within the nucleic acid sequences encoding the CDRs or framework regions, and for incorporation of the resulting modified or fusion nucleic acid sequence into a plasmid for expression. For example, silent substitutions in the nucleotide sequence of the framework and CDR-encoding regions can be used to create restriction enzyme sites which facilitate insertion of mutagenized CDR and/or 10 framework regions. These CDR-encoding regions can be used in the construction of the humanized antibodies of the invention.

The nucleic and amino acid sequences of the 3G9 heavy chain variable region is listed in SEQ ID NO: 1. The CDR amino acid sequences from this region are listed in SEQ ID Nos: 5, 6 and 7.

15 The nucleic and amino acid sequences of the 3G9 light chain variable region listed in SEQ ID NO: 3. The CDR amino acid sequences from this region are listed in SEQ ID Nos: 8, 9 and 10.

Taking into account the degeneracy of the genetic code, various coding 20 sequences may be constructed which encode the variable heavy and light chain amino acid sequences and CDR sequences of the invention as well as functional fragments and analogs thereof which share the antigen specificity of the donor antibody. The isolated nucleic acid sequences of this invention, or fragments thereof, encoding the variable chain peptide sequences or CDRs can be used to produce altered antibodies, *e.g.*, 25 chimeric or humanized antibodies or other engineered antibodies of this invention when operatively combined with a second immunoglobulin partner.

It should be noted that in addition to isolated nucleic acid sequences encoding 30 portions of the altered antibody and antibodies described herein, other such nucleic acid sequences are encompassed by the present invention, such as those complementary to the native CDR-encoding sequences or complementary to the modified human framework regions surrounding the CDR-encoding regions. Useful DNA sequences include those sequences which hybridize under stringent hybridization conditions to the DNA sequences. See, T. Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1982), pp. 387-389. An example of one such stringent 35 hybridization condition is hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for one hour. Alternatively, an exemplary stringent hybridization condition is 50% formamide, 4XSSC at 42°C. Preferably, these hybridizing DNA sequences are at least about 18 nucleotides in length, *i.e.*, about the size of a CDR.

Altered immunoglobulin molecules can encode altered antibodies which include engineered antibodies such as chimeric antibodies and humanized antibodies. A desired altered immunoglobulin coding region contains CDR-encoding regions that encode peptides having the antigen specificity of an EpoR antibody, preferably a high-
5 affinity agonist antibody such as provided by the present invention, inserted into a first immunoglobulin partner such as a human framework or human immunoglobulin variable region.

Preferably, the first immunoglobulin partner is operatively linked to a second immunoglobulin partner. The second immunoglobulin partner is defined above, and
10 may include a sequence encoding a second antibody region of interest, for example an Fc region. Second immunoglobulin partners may also include sequences encoding another immunoglobulin to which the light or heavy chain constant region is fused in frame or by means of a linker sequence. Engineered antibodies directed against functional fragments or analogs of the EpoR may be designed to elicit enhanced
15 binding with the same antibody.

The second immunoglobulin partner may also be associated with effector agents as defined above, including non-protein carrier molecules, to which the second immunoglobulin partner may be operatively linked by conventional means.

Fusion or linkage between the second immunoglobulin partners, *e.g.*, antibody
20 sequences, and the effector agent may be by any suitable means, *e.g.*, by conventional covalent or ionic bonds, protein fusions, or hetero-bifunctional cross-linkers, *e.g.*, carbodiimide, glutaraldehyde and the like. Such techniques are known in the art and are described in conventional chemistry and biochemistry texts.

Additionally, conventional linker sequences which simply provide for a desired
25 amount of space between the second immunoglobulin partner and the effector agent may also be constructed into the altered immunoglobulin coding region. The design of such linkers is well known to those of skill in the art.

In addition, signal sequences for the molecules of the invention may be modified by techniques known to those skilled in the art to enhance expression.

A preferred altered antibody contains a variable heavy and/or light chain peptide or protein sequence having the antigen specificity of mAb 3G9, *e.g.*, the V_H and V_L chains. Still another desirable altered antibody of this invention is characterized by the amino acid sequence containing at least one, and preferably all of the CDRs of the variable region of the heavy and/or light chains of the murine antibody molecule 3G9
30 with the remaining sequences being derived from a human source, or a functional fragment or analog thereof.

In a further embodiment, the altered antibody of the invention may have attached to it an additional agent. For example, recombinant DNA technology may be used to produce an altered antibody of the invention in which the Fc fragment or CH2 CH3 domain of a complete antibody molecule has been replaced by an enzyme or other 5 detectable molecule (*i.e.*, a polypeptide effector or reporter molecule) provided that the dimeric characteristic of the complete antibody molecule is retained.

The second immunoglobulin partner may also be operatively linked to a non-immunoglobulin peptide, protein or fragment thereof heterologous to the CDR-containing sequence having antigen specificity to the EpoR. The resulting protein may 10 exhibit both antigen specificity and characteristics of the non-immunoglobulin upon expression. That fusion partner characteristic may be, *e.g.*, a functional characteristic such as another binding or receptor domain or a therapeutic characteristic if the fusion partner is itself a therapeutic protein or additional antigenic characteristics.

Another desirable protein of this invention may comprise a complete antibody 15 molecule, having full length heavy and light chains or any discrete fragment thereof, such as the Fab or F(ab')2 fragments, a heavy chain dimer or any minimal recombinant fragments thereof such as an F_V or a single-chain antibody (SCA) or any other molecule with the same specificity as the selected donor mAb, *e.g.*, the 3G9 mAb. Such protein may be used in the form of an altered antibody or may be used in its unfused form.

20 Whenever the second immunoglobulin partner is derived from an antibody different from the donor antibody, *e.g.*, any isotype or class of immunoglobulin framework or constant regions, an engineered antibody results. Engineered antibodies can comprise immunoglobulin constant regions and variable framework regions from one source, *e.g.*, the acceptor antibody, and one or more (preferably all) CDRs from the 25 donor antibody, *e.g.*, the 3G9 mAb. In addition, alterations, *e.g.*, deletions, substitutions, or additions, of the acceptor mAb light and/or heavy variable domain framework region at the nucleic acid or amino acid levels, or the donor CDR regions may be made in order to retain donor antibody antigen binding specificity.

Such engineered antibodies are designed to employ one (or both) of the variable 30 heavy and/or light chains of the EpoR mAb (optionally modified as described) or one or more of the heavy or light chain CDRs. The engineered antibodies of the invention exhibit agonist activity.

Such engineered antibodies may include a humanized antibody containing the framework regions of a selected human immunoglobulin or subtype or a chimeric 35 antibody containing the human heavy and light chain constant regions fused to the EpoR mAb functional fragments. A suitable human (or other animal) acceptor antibody may be one selected from a conventional database, *e.g.*, the KABAT®

database, Los Alamos database, and Swiss Protein database, by homology to the nucleotide and amino acid sequences of the donor antibody. A human antibody characterized by a homology to the V region frameworks of the donor antibody or V region subfamily consensus sequences (on an amino acid basis) may be suitable to 5 provide a heavy chain variable framework region for insertion of the donor CDRs. A suitable acceptor antibody capable of donating light chain variable framework regions may be selected in a similar manner. It should be noted that the acceptor antibody heavy and light chains are not required to originate from the same acceptor antibody.

Preferably, the heterologous framework and constant regions are selected from 10 human immunoglobulin classes and isotypes, such as IgG (subtypes 1 through 4), IgM, IgA, and IgE. IgG1, k and IgG4, k are preferred. Particularly preferred is IgG 4, k. Most particularly preferred is the IgG4 subtype variant containing the mutations S228P and L235E (PE mutation) in the heavy chain constant region which results in reduced 15 effector function. This IgG4 subtype variant is known herein as IgG4PE. See U.S. Patent Nos. 5, 624,821 and 5,648,260.

The acceptor antibody need not comprise only human immunoglobulin protein sequences. For instance, a gene may be constructed in which a DNA sequence 20 encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding a non-immunoglobulin amino acid sequence such as a polypeptide effector or reporter molecule.

A particularly preferred humanized antibody contains CDRs of 3G9 mAb inserted onto the framework regions of a selected human antibody sequence. For agonist humanized antibodies, one, two or preferably three CDRs from the 3G9 antibody heavy chain and/or light chain variable regions are inserted into the 25 framework regions of the selected human antibody sequence, replacing the native CDRs of the human antibody.

Preferably, in a humanized antibody, the variable domains in both human heavy and light chains have been engineered by one or more CDR replacements. It is possible to use all six CDRs, or various combinations of less than the six CDRs. Preferably all 30 six CDRs are replaced. It is possible to replace the CDRs only in the human heavy chain, using as light chain the unmodified light chain from the human acceptor antibody. Still alternatively, a compatible light chain may be selected from another human antibody by recourse to conventional antibody databases. The remainder of the engineered antibody may be derived from any suitable acceptor human 35 immunoglobulin.

The engineered humanized antibody thus preferably has the structure of a natural human antibody or a fragment thereof, and possesses the combination of

properties required for effective therapeutic use, *e.g.*, treatment of anemias, cytopenias, acute renal failure and other conditions with depressed erythrocyte production in man.

Most preferably, the humanized antibodies have a heavy chain V region (V_H) amino acid sequence as set forth in SEQ ID NOs: 12 and 14. Also most preferred are 5 humanized antibodies having a light chain V region (V_L) amino acid sequence as set forth in SEQ ID NOs: 16, 18, 20 and 22. Particularly preferred is the humanized antibody 1-0 IgG1,1-0k comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 16. Also particularly preferred is the humanized antibody 1-0 IgG4PE,1-0k comprising a V_H 10 amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 16. Also particularly preferred is the humanized antibody S14 IgG4PE,1-0k comprising a V_H amino acid sequence as set forth in SEQ ID NO: 14 and a V_L amino acid sequence as set forth in SEQ ID NO: 16. Also particularly preferred is the humanized antibody 1-0 IgG1,REIk comprising a V_H amino acid 15 sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 18. Also particularly preferred is the humanized antibody 1-0 IgG4PE,REIk comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 18. Also particularly preferred is the humanized antibody 1-0 IgG1,5-0k comprising a V_H amino acid 20 sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 20. Also particularly preferred is the humanized antibody 1-0 IgG4PE,5-0k comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 20. Also particularly preferred is the humanized antibody 1-0 IgG1,6-0k comprising a V_H amino acid sequence as set forth 25 in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 22. Also particularly preferred is the humanized antibody 1-0 IgG4PE,6-0k comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 22.

It will be understood by those skilled in the art that an engineered antibody may 30 be further modified by changes in variable domain amino acids without necessarily affecting the specificity and high affinity of the donor antibody (*i.e.*, an analog). It is anticipated that heavy and light chain amino acids may be substituted by other amino acids either in the variable domain frameworks or CDRs or both. These substitutions could be supplied by the donor antibody or consensus sequences from a particular 35 subgroup.

In addition, the constant region may be altered to enhance or decrease selective properties of the molecules of this invention. For example, dimerization, binding to Fc

receptors, or the ability to bind and activate complement (see, *e.g.*, Angal *et al.*, *Mol. Immunol.*, 30, 105-108 (1993), Xu *et al.*, *J. Biol. Chem.*, 269, 3469-3474 (1994), Winter *et al.*, EP 307434-B).

An altered antibody which is a chimeric antibody differs from the humanized antibodies described above by providing the entire non-human donor antibody heavy chain and light chain variable regions, including framework regions, in association with human immunoglobulin constant regions for both chains. It is anticipated that chimeric antibodies which retain additional non-human sequence relative to humanized antibodies of this invention may elicit a significant erythropoietic response in humans.

Such antibodies are useful in the prevention of and for treating anemias, cytopenias, acute renal failure and other conditions with depressed erythrocyte production.

Preferably, the variable light and/or heavy chain sequences and the CDRs of mAb 3G9 or other suitable donor mAbs and their encoding nucleic acid sequences, are utilized in the construction of altered antibodies, preferably humanized antibodies, of this invention, by the following process. The same or similar techniques may also be employed to generate other embodiments of this invention.

A hybridoma producing a selected donor mAb, *e.g.*, the murine antibody 3G9, is conventionally cloned and the DNA of its heavy and light chain variable regions obtained by techniques known to one of skill in the art, *e.g.*, the techniques described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory (1989). The variable heavy and light regions of 3G9 containing at least the CDR-encoding regions and those portions of the acceptor mAb light and/or heavy variable domain framework regions required in order to retain donor mAb binding specificity, as well as the remaining immunoglobulin-derived parts of the antibody chain derived from a human immunoglobulin, are obtained using polynucleotide primers and reverse transcriptase. The CDR-encoding regions are identified using a known database and by comparison to other antibodies.

A mouse/human chimeric antibody may then be prepared and assayed for binding ability. Such a chimeric antibody contains the entire non-human donor antibody V_H and V_L regions, in association with human Ig constant regions for both chains.

Homologous framework regions of a heavy chain variable region from a human antibody are identified using computerized databases, *e.g.*, KABAT®, and a human antibody characterized by a homology to the V region frameworks of the donor antibody or V region subfamily consensus sequences (on an amino acid basis) to 3G9 is selected as the acceptor antibody. The sequences of synthetic heavy chain variable regions containing the 3G9 CDR-encoding regions within the human antibody

frameworks are designed with optional nucleotide replacements in the framework regions to incorporate restriction sites. This designed sequence is then synthesized using long synthetic oligomers. Alternatively, the designed sequence can be synthesized by overlapping oligonucleotides, amplified by polymerase chain reaction 5 (PCR), and corrected for errors. A suitable light chain variable framework region can be designed in a similar manner.

A humanized antibody may be derived from the chimeric antibody, or preferably, made synthetically by inserting the donor mAb CDR-encoding regions from the heavy and light chains appropriately within the selected heavy and light chain 10 framework. Alternatively, a humanized antibody of the invention may be prepared using standard mutagenesis techniques. Thus, the resulting humanized antibody contains human framework regions and donor mAb CDR-encoding regions. There may be subsequent manipulation of framework residues. The resulting humanized antibody can be expressed in recombinant host cells, *e.g.*, COS, CHO or myeloma cells.

15 A conventional expression vector or recombinant plasmid is produced by placing these coding sequences for the altered antibody in operative association with conventional regulatory control sequences capable of controlling the replication and expression in, and/or secretion from, a host cell. Regulatory sequences include promoter sequences, *e.g.*, CMV or Rous Sarcoma virus promoter, and signal sequences, 20 which can be derived from other known antibodies. Similarly, a second expression vector can be produced having a DNA sequence which encodes a complementary antibody light or heavy chain. Preferably, this second expression vector is identical to the first except with respect to the coding sequences and selectable markers, in order to ensure, as much as possible, that each polypeptide chain is functionally expressed. 25 Alternatively, the heavy and light chain coding sequences for the altered antibody may reside on a single vector.

30 A selected host cell is co-transfected by conventional techniques with both the first and second vectors (or simply transfected by a single vector) to create the transfected host cell of the invention comprising both the recombinant or synthetic light and heavy chains. The transfected cell is then cultured by conventional techniques to produce the engineered antibody of the invention. The humanized antibody which 35 includes the association of both the recombinant heavy chain and/or light chain is screened from culture by an appropriate assay such as ELISA or RIA. Similar conventional techniques may be employed to construct other altered antibodies and molecules of this invention.

Suitable vectors for the cloning and subcloning steps employed in the methods and construction of the compositions of this invention may be selected by one of skill

in the art. For example, the pUC series of cloning vectors, such as pUC19, which is commercially available from supply houses, such as Amersham or Pharmacia, may be used. Additionally, any vector which is capable of replicating readily, has an abundance of cloning sites and selectable genes (e.g., antibiotic resistance) and is easily manipulated may be used for cloning. Thus, the selection of the cloning vector is not a limiting factor in this invention.

Similarly, the vectors employed for expression of the engineered antibodies according to this invention may be selected by one of skill in the art from any conventional vector. The vectors also contain selected regulatory sequences (such as CMV or Rous Sarcoma virus promoters) which direct the replication and expression of heterologous DNA sequences in selected host cells. These vectors contain the above-described DNA sequences which code for the engineered antibody or altered immunoglobulin coding region. In addition, the vectors may incorporate the selected immunoglobulin sequences modified by the insertion of desirable restriction sites for ready manipulation.

The expression vectors may also be characterized by genes suitable for amplifying expression of the heterologous DNA sequences, e.g., the mammalian dihydrofolate reductase gene (DHFR). Other preferable vector sequences include a poly A signal sequence, such as from bovine growth hormone (BGH) and the betaglobin promoter sequence (betaglopro). The expression vectors useful herein may be synthesized by techniques well known to those skilled in this art.

The components of such vectors, e.g., replicons, selection genes, enhancers, promoters, signal sequences and the like, may be obtained from commercial or natural sources or synthesized by known procedures for use in directing the expression and/or secretion of the product of the recombinant DNA in a selected host. Other appropriate expression vectors of which numerous types are known in the art for mammalian, bacterial, insect, yeast and fungal expression may also be selected for this purpose.

The present invention also encompasses a cell line transfected with a recombinant plasmid containing the coding sequences of the engineered antibodies or altered immunoglobulin molecules thereof. Host cells useful for the cloning and other manipulations of these cloning vectors are also conventional. However, most desirably, cells from various strains of *E. coli* are used for replication of the cloning vectors and other steps in the construction of altered antibodies of this invention.

Suitable host cells or cell lines for the expression of the engineered antibody or altered antibody of the invention are preferably mammalian cells such as CHO, COS, a fibroblast cell (e.g., 3T3) and myeloid cells, and more preferably a CHO or a myeloid cell. Human cells may be used, thus enabling the molecule to be modified with human

glycosylation patterns. Alternatively, other eukaryotic cell lines may be employed. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Sambrook *et al.*, *supra*.

5 Bacterial cells may prove useful as host cells suitable for the expression of the recombinant Fabs of the present invention (see, e.g., Plückthun, A., *Immunol. Rev.*, 130, 151-188 (1992)). However, due to the tendency of proteins expressed in bacterial cells to be in an unfolded or improperly folded form or in a non-glycosylated form, any recombinant Fab produced in a bacterial cell would have to be screened for retention of 10 antigen binding ability. If the molecule expressed by the bacterial cell was produced in a properly folded form, that bacterial cell would be a desirable host. For example, various strains of *E. coli* used for expression are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Streptomyces*, other bacilli and the like may also be employed.

15 Where desired, strains of yeast cells known to those skilled in the art are also available as host cells, as well as insect cells, e.g. *Drosophila* and *Lepidoptera*, and viral expression systems. See, e.g. Miller *et al.*, *Genetic Engineering*, 8, 277-298, Plenum Press (1986) and references cited therein.

20 The general methods by which the vectors of the invention may be constructed, the transfection methods required to produce the host cells of the invention, and culture methods necessary to produce the altered antibody of the invention from such host cell are all conventional techniques. Likewise, once produced, the altered antibodies of the invention may be purified from the cell culture contents according to standard 25 procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Such techniques are within the skill of the art and do not limit this invention.

30 Yet another method of expression of the humanized antibodies may utilize expression in a transgenic animal, such as described in U. S. Patent No. 4,873,316. This relates to an expression system using the animal's casein promoter which when transgenically incorporated into a mammal permits the female to produce the desired recombinant protein in its milk.

35 Once expressed by the desired method, the engineered antibody is then examined for *in vitro* activity by use of an appropriate assay. Presently, conventional ELISA assay formats as well as surface plasmon resonance and isothermal calorimetry are employed to assess qualitative and quantitative binding of the engineered antibody to EpoR. Additionally, other *in vitro* assays such as CFU-E may also be used to determine agonist activity prior to subsequent human clinical studies performed to

evaluate the persistence of the engineered antibody in the body despite the usual clearance mechanisms.

Following the procedures described for humanized antibodies prepared from 3G9, one of skill in the art may also construct humanized antibodies from other donor 5 antibodies, variable region sequences and CDR peptides described herein. Engineered antibodies can be produced with variable region frameworks potentially recognized as "self" by recipients of the engineered antibody. Modifications to the variable region frameworks can be implemented to effect increases in antigen binding and agonist activity without appreciable increased immunogenicity for the recipient. Such 10 engineered antibodies may effectively treat a human for anemias, cytopenias and other conditions with depressed erythrocyte production. Such antibodies may also be useful in the diagnosis of those conditions.

This invention also relates to a method for enhancing erythropoiesis in an animal, particularly a human, which comprises administering an effective dose of an 15 EpoR monoclonal antibody having agonist activity. The mAb can include one or more of the engineered antibodies or altered antibodies described herein or fragments thereof.

In addition, the agonist monoclonal antibodies of the present invention can be co-administered with further active ingredients, such as other compounds known to enhance erythropoiesis or compounds known to have utility when used in combination 20 with an EPO mimetic.

The therapeutic response induced by the use of the molecules of this aspect of the invention is produced by the binding to the EpoR and the subsequent agonist activity of the erythropoietic cascade. Thus, the molecules of the present invention, when in preparations and formulations appropriate for therapeutic use, are highly 25 desirable for persons susceptible to or experiencing anemias, cytopenias and other conditions with depressed erythrocyte production.

This invention also relates to a method for decreasing erythropoiesis in an animal, particularly a human, which comprises administering an effective dose of an EpoR monoclonal antibody having antagonist activity. The mAb can include one or 30 more of the engineered antibodies or altered antibodies described herein or fragments thereof.

In addition, the antagonist monoclonal antibodies of the present invention can be co-administered with further active ingredients, such as other compounds known to decrease erythropoiesis or compounds known to have utility when used in combination 35 with a compound that decreases erythropoiesis.

The therapeutic response induced by the use of the molecules of this aspect of the invention is produced by the binding to the EpoR and the subsequent antagonist

activity of the erythropoietic cascade. Thus, the molecules of the present invention, when in preparations and formulations appropriate for therapeutic use, are highly desirable for persons susceptible to or experiencing conditions with excessive erythrocyte production. Antibodies of this invention may become antagonist of the

5 EpoR under certain circumstances including concentration.

The altered antibodies, antibodies and fragments thereof of this invention may also be used in conjunction with other antibodies, particularly human mAbs reactive with other markers (epitopes) responsible for the condition against which the engineered antibody of the invention is directed.

10 Agonist antibodies to the EPO receptor would have the same therapeutic utility as the natural ligand, but would have the advantage of longer half-life and hence prolonged activity *in vivo*. These agonists can thus be employed to activate the biological cascade which results from receptor/ligand binding. The advantages of 15 EpoR agonist antibodies include the ability to administer lower dosages of antibody than ligand, easier and less frequent administration of a pharmaceutic based on the agonist antibody, as well as easier purification.

15 The EpoR agonist antibodies of the invention can be formulated into pharmaceutical compositions and administered in the same manner as described for mature proteins. See, e.g., International Patent Application, Publication No. 20 WO90/02762 (Mar. 22 1990). Generally, these compositions contain a therapeutically effective amount of an agonist antibody of this invention and an acceptable pharmaceutical carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such compositions may include conventional delivery systems into which protein of the invention is incorporated. 25 Optionally, these compositions may contain other active ingredients, e.g., chemotherapeutics.

20 The therapeutic agents of this invention may be administered by any appropriate internal route, and may be repeated as needed, e.g., as frequently as one to three times daily for between 1 day to about three weeks to once per week or once biweekly. 30 Preferably, the agonist antibody is administered less frequently than is the ligand, when it is used therapeutically. The dose and duration of treatment relates to the relative duration of the molecules of the present invention in the human circulation, and can be adjusted by one of skill in the art depending upon the condition being treated and the general health of the patient.

35 As used herein, the term "pharmaceutical" includes veterinary applications of the invention. The term "therapeutically effective amount" refers to that amount of a receptor agonist antibody, which is useful for alleviating a selected condition. These

therapeutic compositions of the invention may be administered to mimic the effect of the normal receptor ligand.

The mode of administration of the therapeutic agent of the invention may be any suitable route which delivers the agent to the host. The altered antibodies, antibodies, 5 engineered antibodies, and fragments thereof, and pharmaceutical compositions of the invention are particularly useful for parenteral administration, *i.e.*, subcutaneously, intramuscularly, intravenously or intranasally.

Therapeutic agents of the invention may be prepared as pharmaceutical compositions containing an effective amount of the engineered (*e.g.*, humanized) 10 antibody of the invention as an active ingredient in a pharmaceutically acceptable carrier. In the compositions of the invention, an aqueous suspension or solution containing the engineered antibody, preferably buffered at physiological pH, in a form ready for injection is preferred. The compositions for parenteral administration will commonly comprise a solution of the engineered antibody of the invention or a cocktail 15 thereof dissolved in an pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, *e.g.*, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary 20 substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antibody of the invention in such pharmaceutical formulation can vary widely, *i.e.*, from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of 25 administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and between about 1 ng to 30 about 100 mg, *e.g.* about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of an engineered antibody of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 mg to about 30 mg and preferably 35 5 mg to about 25 mg of an engineered antibody of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, "Remington's Pharmaceutical Science", 15th ed., Mack Publishing Company, Easton, Pennsylvania.

It is preferred that the therapeutic agent of the invention, when in a pharmaceutical preparation, be present in unit dose forms. The appropriate

therapeutically effective dose can be determined readily by those of skill in the art. To effectively treat anemia in a human or other animal, one dose of approximately 0.01 mg to approximately 20 mg per kg body weight of a protein or an antibody of this invention should be administered parenterally, preferably *i.v.* or *i.m.* Such dose may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician during the response period.

5 Optionally, the pharmaceutical compositions of the invention may contain other active ingredients or be administered in conjunction with other therapeutics. Suitable optional ingredients or other therapeutics include those conventional for treating 10 conditions of this nature, e.g. EPO or other agents known for the treatment of anemias, cytopenias and other conditions with depressed erythrocyte production.

The present invention will now be described with reference to the following specific, non-limiting examples.

15

Example 1

Preparation and Screening of EpoR Agonist Monoclonal Antibodies

Monoclonal Antibody Generation

20 Mice (F1 hybrids of Balb/c and C57BL/6) were immunised subcutaneously with 10 ug recombinant EpoR in Freunds complete adjuvant and 4 weeks later with 10ug EpoR in Freunds incomplete adjuvant. On the basis of a good serum antibody titer to EpoR, one mouse received further immunization of 25 ug EpoR (i.p. in saline) at 8 weeks and another similar immunization two days later. A splenectomy was performed 25 two days following the final immunization. Mouse spleen cells were used to prepare hybridomas by standard procedures, (Zola, H.Ed., Monoclonal Antibodies, CRC Press Inc. (1987)). Positive hybridomas were cloned by the limiting dilution method.

Hybridoma Screening Assay

30 96-well plates were coated with EpoR-Fc (0.5ug/ml, 100ul/well in PBS) by incubation overnight at 4°C. The solution was then aspirated and non-specific binding sites were blocked with 250ul/well of 1% bovine serum albumin (BSA) in TBS buffer (50mM Tris, 150 mM NaCl, 0.02% Kathon, pH 7.4) for 5-60 minutes at RT. Following this and each of the following steps, the plate was washed 4 times in wash 35 buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.02% Kathon, pH 7.4). To each well, 50 uL hybridoma medium and 50 uL assay buffer (0.5% BSA, 0.05% bovine gamma globulin, 0.01% Tween 40, 20uM diethylenetriaminepentaacetic in TBS buffer)

was added and the plates were incubated for 60 min at RT in a shaker-incubator, followed by an incubation of 60 min at RT in a shaker-incubator with 100ul 0.5ug/ml Eu³⁺-labelled anti-mouse antibody in assay buffer. Finally, 100 ul /well of enhancer (Wallac) was added and incubated for 5 min at RT and the fluorescence measured.

5 Hybridomas having counts >500K were expanded into 24-well plates.

Immunoassay

To determine the specificity of the anti-EpoR mAbs generated, 96-well plates were coated (0.5ug/ml EpoR-Fc, 100ul/well) and blocked as above. All the following 10 incubations were performed in a shaker-incubator at RT. After washing the wells 50ul EpoR (3ug/ml) or 50ul assay buffer and 50ul mAb were added and incubated for 60 min. After washing the wells 100ul 0.5ug/ml Eu³⁺ labelled anti-mouse antibody in assay buffer was added for 60 min, the wells washed and then 100 ul /well of enhancer (Wallac) was added and incubated for 5 min at RT and the fluorescence measured. All 15 positive hybridomas, including 3G9, showed displacement of binding with EpoR.

Selection of Antibodies by Flow Cytometry on UT7-Epo cells

Flow cytometry was used to select hybridomas and primary clones that bind to the external domain of the wild type EpoR. A human megakaryoblastic cell line 20 selected in Epo, UT-7-Epo, expresses the Epo receptor on its cell surface.

The mean and median values of fluorescent intensity were measured. Mean fluorescence is the average fluorescent intensity of a population of cells and the median intensity is the middle value between two extremes within the population. The fluorescence of 10,000 cells from each sample was measured. Monoclonal antibody 25 3G9, which bound to cell surface erythropoietin receptors, showed enhanced fluorescence over background and control monoclonal antibodies.

Purification of Mabs

Monoclonal antibodies were purified by ProsepA (Bio Processing Inc., 30 Princeton, NJ) chromatography respectively per the manufacturer's instructions. Mabs were >95% pure by SDS-PAGE.

Example 2

Biophysical characterization of EpoR Agonist Monoclonal Antibodies

35

Competition for Binding to EpoR with Epo

Antibodies were assessed for their ability to compete with Epo for binding to EpoRFc by surface plasmon resonance using a BIACore instrument. Refractive index units (RU) increased for the sequential addition of EpoRFc, Epo and monoclonal antibody (or buffer), or the sequential addition of EpoRFc, mAb (or buffer) and Epo.

5 The RU are a direct measure of the amount of each protein which can bind. Hence, prebinding of Epo reduces the amount of 3G9 which can bind. Similarly, prebound 3G9 is displaced by Epo which results in a negative change in RU.

Goat anti-human IgG, Fc specific antibody was immobilised on a sensor chip surface and 25ul Epo-Rec-Fc (2ug/ml diluted in HBS buffer) at 5ul/min. was injected, 10 the RU recorded, followed by injections of 25ul Epo (5ug/ml diluted in HBS buffer), record RU and 25ul 3G9 Mab (10ug/ml in HBS buffer), record RU. The surface was regenerated with an injection of 15ul 0.1M phosphoric acid. The above was repeated reversing the order of addition for Epo and 3G9 Mab. These data showed that the 15 monoclonal antibody 3G9 competed with Epo for binding to the erythropoietin receptor.

Affinity Measurements of 3G9 Monoclonal Antibody

The affinity of 3G9 was measured in the BIACore. Using a flow rate of 5ul/min, Mab 3G9 (diluted in HBS buffer) was injected over a rabbit anti-mouse Fc surface, 20 followed by buffer flow and the RU recorded. EpoR or EpoRFc diluted in HBS buffer at 0.25-6 ug/ml was then injected for 120 s followed by buffer flow for 240 s and regeneration of the sensor chip surface with an injection of 15 ul 0.1 M phosphoric acid. BIACore software was used for association and dissociation-phase analysis.

The parent murine monoclonal antibody, 3G9, and humanized and chimeric 25 derivatives bound to soluble monomeric erythropoietin receptor (EpoR) with an on-rate (k_{ass}) of $1.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and an off-rate (k_{diss}) of $1.1 \times 10^{-3} \text{ s}^{-1}$. Together, these yield a calculated equilibrium constant (K_D) of 10 nM. The parent murine monoclonal antibody, 3G9, and humanized and chimeric derivatives bound to soluble dimeric erythropoietin receptor (EpoRFc) with an on-rate (k_{ass}) of $3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and an off-rate (k_{diss}) of $1.9 \times 10^{-3} \text{ s}^{-1}$. Together, these yield a calculated equilibrium constant (K_D) 30 of 0.6nM.

Example 3

Biological Activity of EpoR Monoclonal antibodies

35 **Self-Limiting Effect**

UT7-Epo proliferation

UT-7Epo is a human cell line which depends on Epo for growth. Thymidine incorporation was used to measure proliferation of UT7-Epo cells. 5×10^4 cells in log phase growth were plated in 100 μ l IMDM/10% FCS per well of a 96-well microtiter plate with test samples and Epo control curve. After a 3 day incubation at 37°C, 3 H-thymidine (1 uCi/well; NEN) was added for 4 hrs and the plate harvested with TCA and cold ethanol. Solid scintillant (Meltilex; Wallac) was melted onto the filter containing the samples and radioactivity measured on a Betaplate reader (Wallac). Data were reported in Fig. 1 as the mean of quadruplicate samples.

The 3G9 mAb stimulated greater proliferative activity than the Epo control. Maximum proliferative activity was at 0.3 μ g/ml and there was a bell-shaped dose response curve as concentration increased. The negative control antibody 3B3 had no activity in this assay.

Human CFU-E

Light density cells from human bone marrow centrifuged over Histopaque 1077 (Sigma) were washed and resuspended at 2.5×10^6 cells/ml in X-vivo medium (Biowhittaker). The purified monoclonal antibodies were diluted in X-vivo medium, and the Epo positive control was 2 U/ml for the assay, 0.3 ml cells, 0.3 ml mAb sample (or Epo control) and 0.7 ml X-vivo medium were incubated in a polypropylene tube for 30 min at RT, then 0.9 ml FCS, 0.3 ml 10% BSA and 0.8 ml 3.2% methylcellulose were added. 0.4 ml were plated per well of a 24-well TC dish (Nunc). Colonies were identified microscopically as more than 8 red, hemoglobinized cells scored at day 7.

The results in Fig. 2 show that purified 3G9 mAb was most active at 0.3 μ g/ml and has a bell-shaped dose response curve. The negative control antibody 3B3 had no significant activity.

The results in Fig. 3 show that the 3G9 humanized REI construct 1-0 IgG4PE,REIk (Hz REI g4) expressed in CHO stimulated 52% of the number of colonies as a maximal amount of Epo. The humanized 3G9 pro to ser mutant S14 IgG4PE,1-0k (Hz Pro-Ser g4) expressed in COS cells had an equivalent number of colonies as the murine mAb (Mu3G9), 36 and 38% of Epo, respectively.

The HL5 humanized 3G9 construct 1-0 IgG1,5-0k had activity equal or greater than the murine 3G9 monoclonal antibody (57-68% of Epo control for HL5 vs. 55-58% for murine 3G9) in the human CFU-E assay (data not shown). The HL6 humanized 3G9 construct 1-0 IgG1,6-0k had approximately 25% of the Epo control activity in human CFU-E (data not shown).

Cross reactivity of an anti-human EpoR monoclonal antibody with various non-human EpoRs can allow the evaluation of 3G9 *in vivo* in the corresponding animal.

Primate CFU-E

5 Primate marrow was prepared in the same way as human marrow. Marrow cells obtained from cynomolgus macaques were centrifuged on Histopaque 1066, washed and resuspended to 2.5×10^6 cells/ml. Epo control was 2 U/ml. The cells and antibody samples were incubated similarly, FCS, BSA and methylcellulose added and plated.

10 Colonies were scored at day 7. Fig. 4 shows that the humanized 3G9 REI construct 1-0 IgG4PE,REIk (Hz REI gamma4) stimulated as many colonies as the maximal Epo control. The humanized 3G9 pro to ser mutant S14 IgG4PE,1-0k had an equivalent amount of CFU-E colonies stimulated as the murine 3G9 antibody (data not shown).

Rabbit CFU-E

15 Rabbit marrow was flushed from the femur, washed and resuspended to 2.5×10^6 cells/ml. The cells were not centrifuged through Histopaque before addition to the antibodies. All other components and methods were similar to the human marrow. Fig. 5 shows that 3G9 had maximal activity at 0.3 ug/ml, with many more colonies than seen with Epo. The negative control antibody 3B3, which also binds to EpoR, had 20 fewer colonies than the negative control.

Rabbit Reticulocyte Model

New Zealand White rabbits were injected i.v. with a single dose of 1 or 5 mg/kg murine 3G9 mAb, or i.v. with Epo (100 U/kg) 3 times per week. Blood samples 25 were taken and reticulocytes were counted on a Sysmex reticulocytometer. As shown in Table 1 below 5 mg/kg murine 3G9 mAb elevated reticulocytes on day 5 significantly above the control.

Table 1 - Effects on Rabbit Reticulocytes

	Reticulocytes ($10^9/L$) Pre-dose	Reticulocytes ($10^9/L$) Day 5	fold increase in reticulocytes
Control	138.0	136.4	0.99
Epo (100 U/kg)	111.7	341.8	3.05
Mu 3G9 mAb (5 mg/kg)	135.9	200.1	1.47

Intracellular signaling

Upon binding to its receptor, Epo stimulates the activation of an EpoR bound tyrosine kinase, JAK, through tyrosine phosphorylation, and the tyrosine 5 phosphorylation of a latent cytoplasmic transcription factor, STAT5. Upon tyrosine phosphorylation, STAT5 translocates to the nucleus, and binds to regulatory regions of DNA, resulting in transcriptional activity of the associated gene. JAK activation was measured by immunoprecipitation with anti-JAK2 antibody and western blotting with anti-phosphotyrosine. UT7-Epo cells were grown in IMDM/10% FCS and starved of 10 Epo for 24 hrs. The cells were then treated with Epo (0.1 and 1 U/ml) or monoclonal antibody 3G9 or 3B3 (0.003-3 ug/ml) for 10 min. After pelleting the cells, lysis buffer was added (0.05 M Tris-HCl, 1 mM sodium vanadate, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM Pefabloc, 10 ug/ml aprotinin, 10 ug/ml leupeptin), and the samples incubated on ice for 20 min with occasional vortexing, after which the samples 15 are centrifuged 1800 rpm for 3 min, 4°C and the supernatants collected. Protein determinations were made with the BCA protein assay (Pierce, Arlington Heights, Illinois).

22.5 ug of each lysate was immunoprecipitated with 15 ug of agarose-conjugated JAK2 (UBI) for 1.5 hr at 4°C, centrifuged, and the pellet washed two times 20 in cold lysis buffer. The pellet was then resuspended in SDS Tris-glycine sample buffer with 2.5% 2-mercaptoethanol and 20 ul run on a 8% Tris glycine gel. The samples were transferred to PVDF membranes and western blotted with anti-phosphotyrosine (1 ug/ml) for 1 hr using 0.5% gelatin/PBS-Tween-20 as the blocking buffer, HRP-labeled goat anti-mouse (Amersham, Rockford, Illinois) secondary 25 antibody for 1 hr and detection using the enhanced chemiluminescence (ECL) reagents (Amersham).

As shown in Fig. 6 below, 3G9 mAb induced activation (tyrosine-phosphorylation) of JAK2 with peak activation at 0.3 ug/ml mAb was equivalent to 50% of the maximum activation induced by Epo (1 U/ml). 3B3 did not activate JAK2.

30

Example 4

Cloning and Sequencing of 3G9 Light and Heavy Chain cDNAs

The amino acid sequences of 13 light chain amino-terminal residues and 15 heavy chain amino-terminal residues of 3G9 were determined. The amino terminus of 35 the heavy chain was blocked with pyroglutamic acid. It was successfully deblocked enzymatically using pyroglutamate aminopeptidase.

Total 3G9 RNA was purified, reverse transcribed and PCR amplified. For the heavy chain, the RNA/DNA hybrid was PCR amplified using a mouse IgG1 hinge primer and a degenerate primer based on the N-term protein sequence. Similarly, for the light chain, the RNA/DNA hybrid was PCR amplified using a mouse kappa primer and a degenerate primer based on the N-term protein sequence. PCR inserts of the appropriate size, *i.e.*, ~700 bp for the heavy chain and ~400 bp for the light chain were sequenced by a modification of the Sanger method. The sequence of 5 heavy and 4 light chain clones were compared to generate a consensus 3G9 heavy chain variable region sequence (SEQ ID NO: 1) and consensus 3G9 light chain variable region sequence (SEQ ID NO: 3). The heavy chain CDR 1, 2 and 3 amino acid sequences are shown in SEQ ID NOs: 5, 6 and 7, respectively. The light chain CDR 1, 2 and 3 amino acid sequences are shown in SEQ ID NOs: 8, 9 and 10, respectively.

Example 5

Humanization of the 3G9 Antibody

Six humanized V region constructs were designed to contain the murine CDRs described above in a human antibody framework. In each case, the humanized V_H and V_L regions were first cloned into pCR2000 shuttle vectors, sequenced, corrected for mistakes, and then transferred to expression vectors as AgeI/KpnI and AgeI/ApaI fragments for V_L and V_H regions, respectively. The final humanized expression constructs encode complete heavy and light chains, comprising the initiation codon and the end of the C_H and C_H^3 domains of the heavy and light chains, respectively.

1-0 IgG1,1-0k

The humanized antibody 1-0 IgG1,1-0k contains the heavy chain V region 3G9HZHC 1-0 and the light chain V region 3G9HZLC 1-0.

The synthetic humanized heavy chain V region 3G9HZHC 1-0 was designed using the homologous framework of the human V_H subgroup I consensus sequence, generated from Kabat database sequences, and the 3G9 murine heavy chain CDRs described previously. Eight framework amino acids, which were predicted to influence CDR presentation, were substituted with the corresponding murine 3G9 residues. The construct 3G9HZHC1-0 includes the complete V_H region and its sequence is shown in SEQ ID NO: 11.

The synthetic humanized light chain V region 3G9HZLC 1-0 was designed using the human kappa subgroup 1 framework consensus sequence and the 3G9 murine light chain CDRs described above. Three framework amino acids, which were predicted to influence CDR presentation, were substituted with the corresponding

murine 3G9 residues. The construct 3G9HZLC 1-0 includes the complete V_L region and its sequence is shown in SEQ ID NO: 15.

1-0 IgG4PE,1-0k

5 The humanized antibody 1-0 IgG4PE,1-0k contains the heavy chain V region 3G9HZHC 1-0 (SEQ ID NO: 11) and the light chain V region 3G9HZLC 1-0 (SEQ ID NO: 15). 3G9HZHC 1-0 (SEQ ID NO: 11) was inserted into an IgG4PE mutation expression vector.

S14 IgG4PE,1-0k

10 The humanized antibody S14 IgG4PE,1-0k contains the heavy chain V region 3G9HZHC S14 and the light chain V region 3G9HZLC 1-0 (SEQ ID NO: 15).

A variant of 3G9HZHC 1-0 (SEQ ID NO: 11) was constructed containing a serine residue substituted for proline in the V_H region at position 14. The sequence of the construct 3G9HZHC S14 is shown in SEQ ID NO: 13.

15 **1-0 IgG1,REIk**

The humanized antibody 1-0 IgG1,REIk contains the heavy chain V region 3G9HZHC 1-0 (SEQ ID NO: 11) and the light chain V region 3G9HZLC 1-0REI.

20 A variant of 3G9HZLC 1-0 (SEQ ID NO: 15) was constructed using the framework residues of a derivative of the human light chain REI, REI-con (SEQ ID NO: 23). The framework of REI is very similar to that of the human kappa subgroup I consensus sequence used above for the construction of 3G9HZLC 1-0. In fact, only two residues of 3G9HZLC 1-0 were changed to generate 3G9HZLC-REI. Accordingly, as for 3G9HZLC 1-0, three framework amino acids, which were predicted to influence CDR presentation, were substituted with the corresponding murine 3G9 residues. The 25 construct 3G9HZLC 1-0REI includes the complete V_L region and its sequence is shown in SEQ ID NO: 17.

1-0 IgG4PE,REIk

30 The humanized antibody 1-0 IgG4PE,REIk contains the heavy chain V region HZHC 1-0 (SEQ ID NO: 11) inserted into the IgG4PE expression vector and the light chain V region HZLC 1-0REI (SEQ ID NO: 17).

1-0 IgG1,5-0k

The humanized antibody 1-0 IgG1,5-0k contains the heavy chain V region HZHC 1-0 (SEQ ID NO: 11) and the light chain V region HZLC 5-0.

35 A variant of HZLC 1-0 (SEQ ID NO: 15) was constructed by site directed mutagenesis of HZLC 1-0 in which a single residue (Phe73) of the framework of a derivative of the human light chain REI, REI-con (SEQ ID NO 23) was introduced at

position V_L 73. The construct HZLC5-0 includes the complete V_L region and its sequence is shown in SEQ ID NO: 19.

1-0 IgG4PE,5-0k

5 The humanized antibody 1-0 IgG4PE,5-0k contains the heavy chain V region HZHC 1-0 (SEQ ID NO: 11) inserted into the IgG4PE expression vector and the light chain V region HZLC 5-0 (SEQ ID NO: 19).

1-0 IgG1,6-0k

10 The humanized antibody 1-0 IgG1,6-0k contains the heavy chain V region HZHC 1-0 (SEQ ID NO: 11) and the light chain V region HZLC 6-0.

15 A variant of HZLC 1-0 (SEQ ID NO: 15) was constructed by site directed mutagenesis of HZLC 1-0 in which a single residue (Ile83) of the framework of a derivative of the human light chain REI, REI-con (SEQ ID NO 23) was introduced at position VL83. The construct HZLCLC 6-0 includes the complete V_L region and its sequence is shown in SEQ ID NO: 21.

1-0 IgG4PE,6-0k

20 The humanized antibody 1-0 IgG4PE,6-0k contains the heavy chain V region HZHC 1-0 (SEQ ID NO: 11) inserted into the IgG4PE expression vector and the light chain V region HZLC 6-0 (SEQ ID NO: 21).

Example 6

Expression of Humanized 3G9 Antibodies in Mammalian Cells

25 The humanized heavy and light chains described above were expressed in expression plasmid derivatives of pCDN (A. Nambi, et al., (1994), *Mol. Cell. Biochem.*, 131:75-85). Accordingly, each expression plasmid variant contains, in general, a beta-lactamase gene, an SV40 origin of replication, a cytomegalovirus promoter sequence, a selected humanized heavy or light chain, a poly A signal for bovine growth hormone (BGH), a betaglobin promoter, a dihydrofolate reductase gene, and another BGH sequence poly A signal. These features are present in a pUC19 background for 30 bacterial replication of the plasmid.

For initial characterization, the humanized 3G9 constructs were transiently expressed in COS cells essentially as described in Current Protocols in Molecular Biology (edited by F. M. Ausubel et al. (1988), John Wiley and Sons, vol. I, section 9.1). Briefly, COS cells were co-transfected with 10 micrograms each of heavy and 35 light chain expression construct. After one day of culture, the growth medium was replaced with serum free medium, which was harvested and replaced on day three. Culture supernatant was again harvested on day five, and reserved for further analysis.

Example 7
3G9 anti-EpoR antagonist antibody

Fig. 7

5 **Murine 3G9 mAb competes with 125 I-labeled Epo for binding to UT7-Epo cells.**
1 nM 125 I-labeled Epo (Amersham) and different concentration of 3G9 mAb or excess
cold Epo were added simultaneously to 5×10^5 UT7-Epo cells. Following 5 hrs
incubation at 4°C, the cells were spun through horse serum, frozen, and the pellets
clipped off and radioactivity counted. Murine 3G9 antibody at 0.03-30 ug/ml inhibited
10 Epo binding greater than 90%.

Fig. 8

Inhibition of Epo-stimulated CFU-E by the presence of Hz3G9 mAb

Hz3G9 antibody at 30 ug/ml inhibited Epo-stimulated human CFU-E at Epo
15 concentrations of 0.005-100 U/ml. Hz3G9 was added to the marrow cell mix at the
same time as Epo.

Fig. 9

Inhibition of cynomolgus monkey hematocrit by single i.v. dose of Hz3G9.

20 Hematocrit was measured following a single i.v. dose of Hz3G9 at 0.1, 0.5, 1 and 5
mg/kg into cynomolgus macaques. A dose dependent decrease in hematocrit was
evident as early as day 10. The duration of hematocrit decrease was also dose
dependent and lasted up to 13 weeks at the 5 mg/kg dose.

The present invention may be embodied in other specific forms without
25 departing from the spirit or essential attributes thereof, and, accordingly, reference
should be made to the appended claims, rather than to the foregoing specification, as
indicating the scope of the invention.

CLAIMS

1. A method for enhancing erythropoiesis in an animal comprising administering an effective dose of an erythropoietin receptor agonist antibody having the identifying characteristics of monoclonal antibody 3G9; 1-0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k, 1-0 IgG1,6-0k; or 1-0 IgG4PE,6-0k.
2. The method of claim 1 further comprising administering an additional active ingredient in combination with the antibody.
3. The method of claim 1 or 2 wherein the subject is in need of treatment for anemias, cytopenias or acute renal failure.
4. An EpoR agonist antibody having the identifying characteristics of monoclonal antibody 3G9; 1-0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k, 1-0 IgG1,6-0k; or 1-0 IgG4PE,6-0k.
5. A hybridoma having the identifying characteristics of cell line 3G9.
6. An EpoR agonist antibody comprising a heavy chain variable region (V_H) amino acid sequence as set forth in SEQ ID NO: 2 and a light chain variable region (V_L) amino acid sequence as set forth in SEQ ID NO: 4.
7. The EpoR agonist antibody of claim 6 which is monoclonal antibody 3G9.
8. An EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 16.
9. The EpoR agonist antibody of claim 8 which is 1-0 IgG1,1-0k.
10. The EpoR agonist antibody of claim 8 which is 1-0 IgG4PE,1-0k.
11. An EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 14 and a V_L amino acid sequence as set forth in SEQ ID NO: 16.
12. The EpoR agonist antibody of claim 11 which is S14 IgG4PE,1-0k.
13. An EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 18.
14. The EpoR agonist antibody of claim 13 which is 1-0 IgG1,REIk.
15. The EpoR agonist antibody of claim 13 which is 1-0 IgG4PE,REIk.
16. An EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 20.
17. The EpoR agonist antibody of claim 16 which is 1-0 IgG1,5-0k.

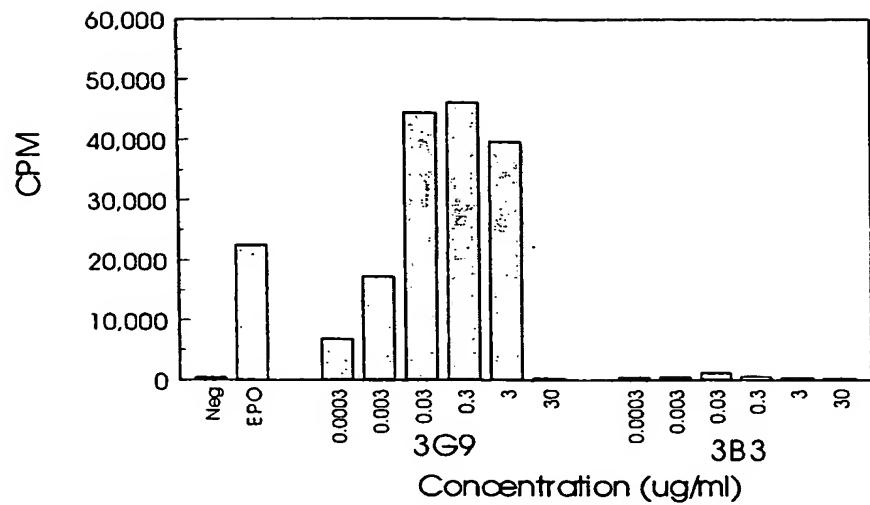
18. The EpoR agonist antibody of claim 16 which is 1-0 IgG4PE,5-0k.
19. An EpoR agonist antibody comprising a V_H amino acid sequence as set forth in SEQ ID NO: 12 and a V_L amino acid sequence as set forth in SEQ ID NO: 22.
20. The EpoR agonist antibody of claim 19 which is 1-0 IgG1,6-0k.
- 5 21. The EpoR agonist antibody of claim 19 which is 1-0 IgG4PE,6-0k.
22. An EpoR agonist antibody comprising a V_H amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 12 or 14.
23. An EpoR agonist antibody comprising a V_L amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 16, 18, 20 or 22.
- 10 24. The EpoR agonist antibody of claim 22 or 23 wherein the heavy chain constant regions are immunoglobulin class IgG, IgM, IgA or IgE.
25. The EpoR agonist antibody of claim 24 wherein the heavy chain constant regions are subtype IgG1, IgG2, IgG3 or IgG4.
- 15 26. The EpoR agonist antibody of claim 25 wherein the heavy chain constant region is an IgG4 subtype and contains S228P and L235E mutations.
27. A bivalent fragment of the antibody of claim 24.
28. An immunoglobulin heavy chain complementarity determining region, the amino acid sequence of which is selected from the group consisting of SEQ ID NOs: 5, 6 and 7.
- 20 29. An isolated nucleic acid molecule encoding the immunoglobulin complementarity determining region of claim 28.
30. An immunoglobulin light chain complementarity determining region, the amino acid sequence of which is selected from the group consisting of SEQ ID NOs: 8, 9 and 10.
31. An isolated nucleic acid molecule encoding the immunoglobulin complementarity determining region of claim 30.
- 25 32. An altered antibody comprising a heavy chain and a light chain, wherein the framework regions of said heavy and light chains are derived from at least one selected antibody and the amino acid sequences of the complementarity determining regions of each said chain are derived from the antibody of claim 7.
33. The altered antibody of claim 32 which is humanized.
- 30 34. The altered antibody of claim 32 which is human.
35. A chimeric EpoR agonist antibody comprising a heavy chain and a light chain derived from at least one selected antibody and the amino acid sequences of the V_L and V_H regions are derived from the antibody of claim 7.

36. An isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NOs: 2, 4, 12, 14, 16, 18, 20 or 22 and functional fragments or analogs therof.

37. A pharmaceutical composition comprising the antibody of claim 4, 26 or 32 and a pharmaceutically acceptable carrier.

5 38. The pharmaceutical composition of claim 37 further comprising an additional active ingredient.

39. A method for decreasing erythropoiesis in an animal comprising administering an effective dose of an erythropoietin receptor antagonist antibody having the identifying characteristics of monoclonal antibody 3G9; 1-0 IgG1,1-0k; 1-0 IgG4PE,1-0k; S14 IgG4PE,1-
10 0k; 1-0 IgG1,REIk; 1-0 IgG4PE,REIk; 1-0 IgG1,5-0k; 1-0 IgG4PE,5-0k, 1-0 IgG1,6-0k; or 1-0 IgG4PE,6-0k.

**Figure 1****1/9**

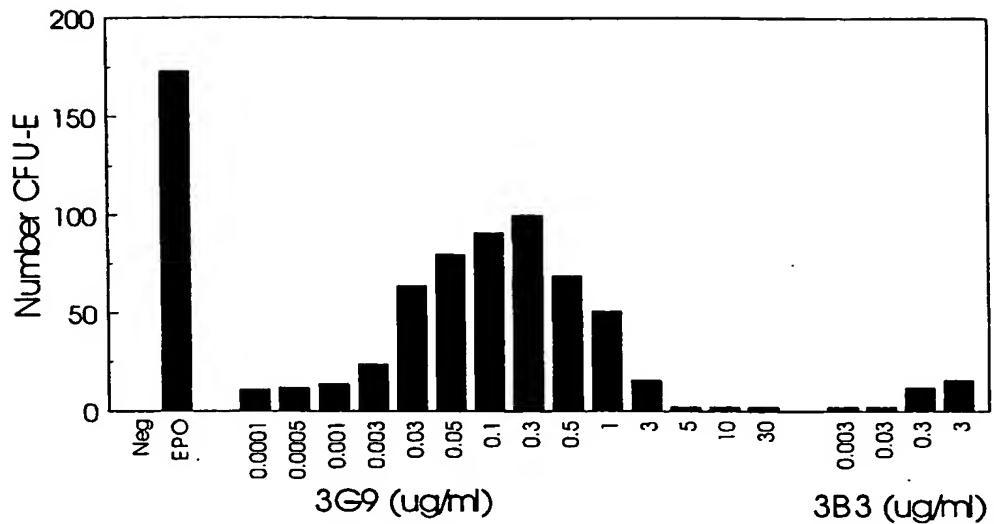


Figure 2

2/9

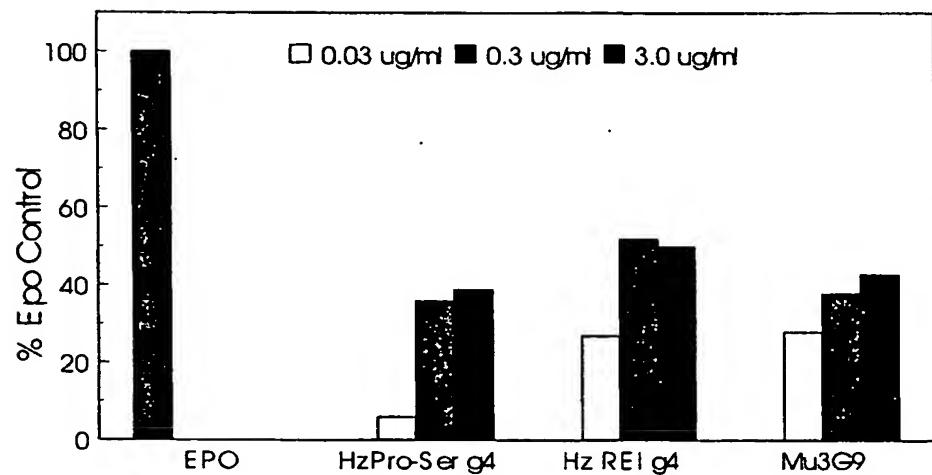


Figure 3

3/9

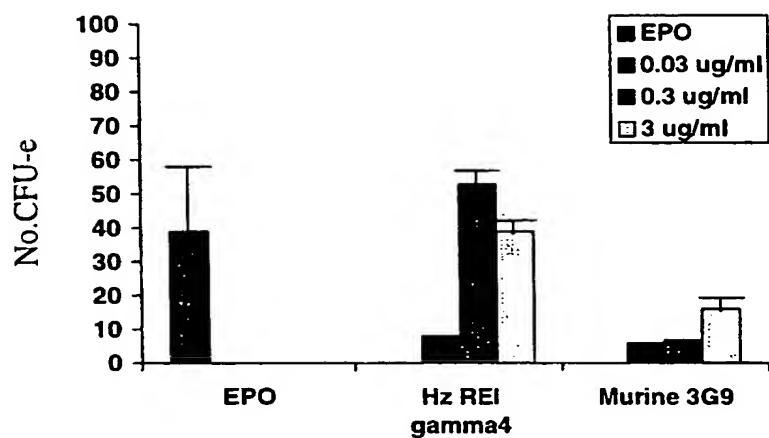


Figure 4

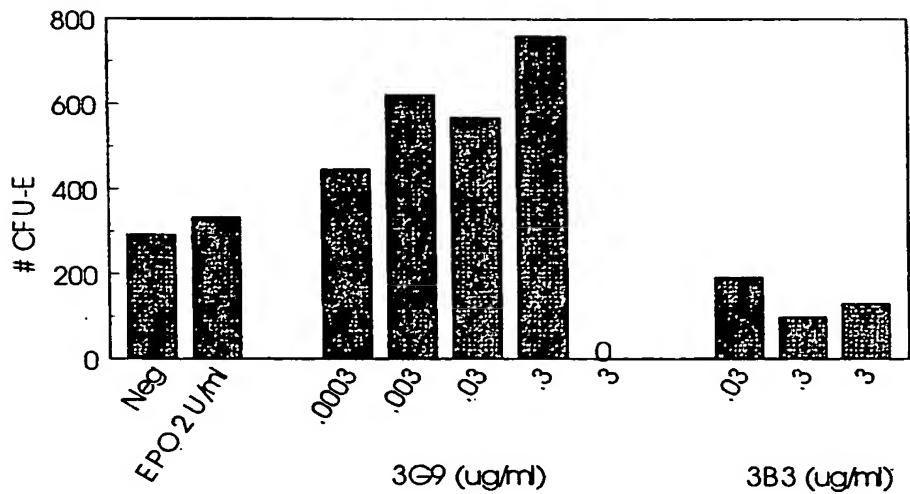


Figure 5

5/9

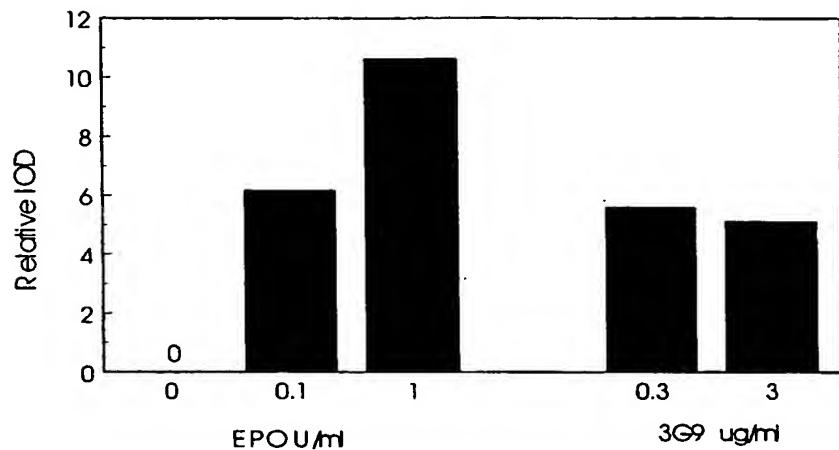


Figure 6

6/9

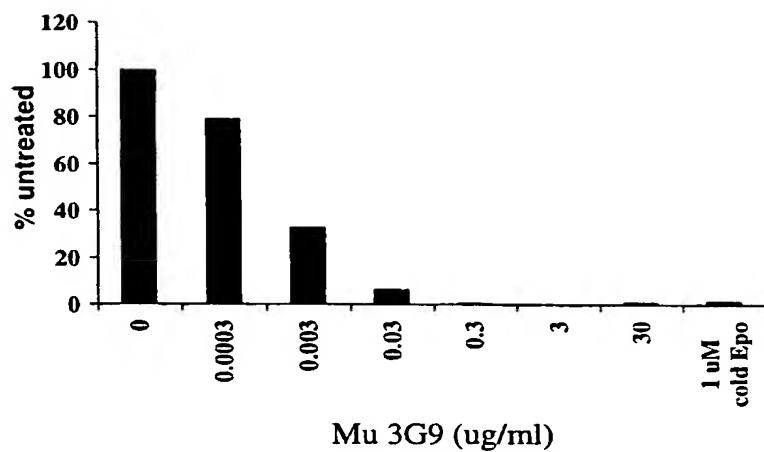


Figure 7

7/9

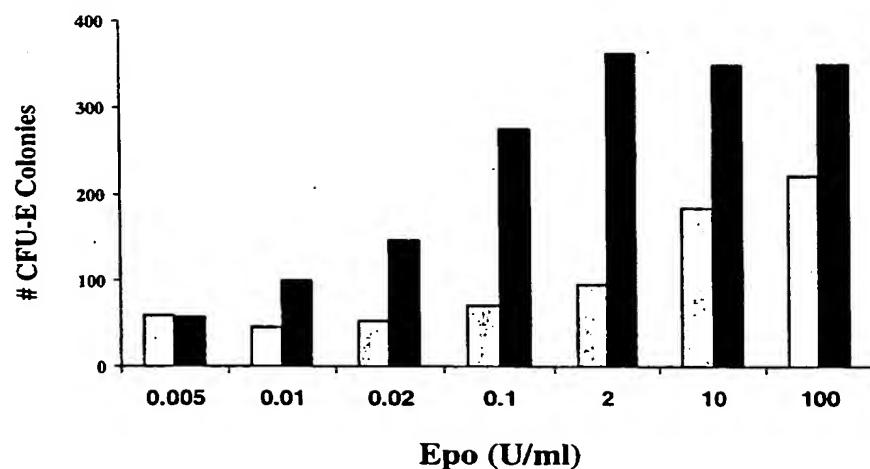
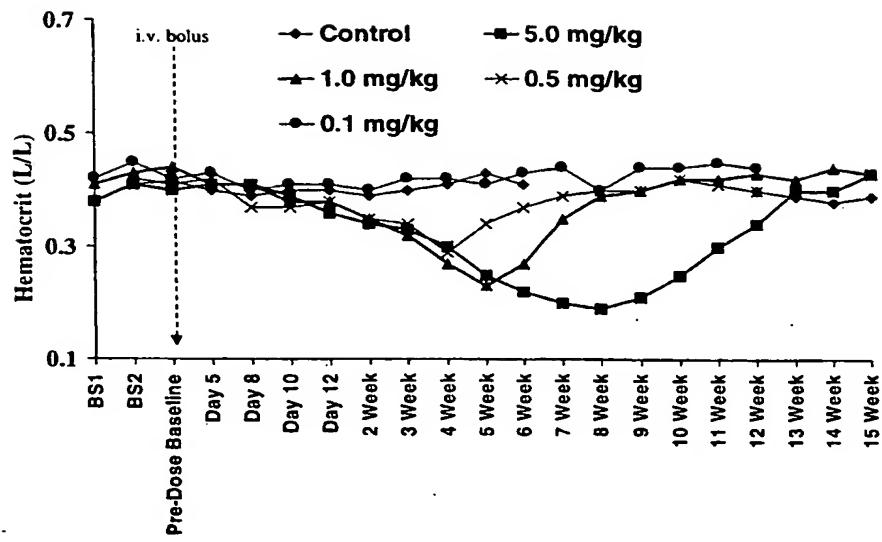


Figure 8

8/9

**Figure 9**

9/9

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Erickson-Miller, Connie L
Holmes, Stephen D
Taylor, Alexander H
Young, Peter R

(ii) TITLE OF THE INVENTION: Erythropoietin Receptor Antibodies

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: SmithKline Beecham Corporation
(B) STREET: 709 Swedeland Road
(C) CITY: King of Prussia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19406

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: UNKNOWN
(B) FILING DATE: 14-APR-2000
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/129,263
(B) FILING DATE: 14-APR-1999

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Baumeister, Kirk
(B) REGISTRATION NUMBER: 33,833
(C) REFERENCE/DOCKET NUMBER: P50911

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 610-270-5096
(B) TELEFAX: 610-270-5090
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: 3G9 VH region
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...360
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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48

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96

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144

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65	70	75	80
ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAT TGT	288		
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys			
85	90	95	
GCA AGA GAG ACC TAC TAT GAT TCC TCG TTT GCT TAC TGG GGC CAA GGG	336		
Ala Arg Glu Thr Tyr Tyr Asp Ser Ser Phe Ala Tyr Trp Gly Gln Gly			
100	105	110	
ACT CTG GTC ACT GTC TCT GCA GCC	360		
Thr Leu Val Thr Val Ser Ala Ala			
115	120		

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 120 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9 VH region

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Ser Gly Ala			
1	5	10	15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Ser Thr Phe Thr Ser Tyr
 20 25 30

Trp Met His Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
 35 40 45

Gly Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys Asp Asn Glu Lys Phe
 50 55 60

Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Pro Ser Ser Thr Ala Tyr
 65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
 85 90 95

Ala Arg Glu Thr Tyr Tyr Asp Ser Ser Phe Ala Tyr Trp Gly Gln Gly
 100 105 110

Thr Leu Val Thr Val Ser Ala Ala
 115 120

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9 VL region

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence

- (B) LOCATION: 1...336

- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAT ATT GTT ATG ACT CAG TCT CAA AAA TTC ATG TCC ACA TCA GTA GGA
 Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

48

GAC AGG GTC AGC GTC ACC TGC AAG GCC AGT CAG AAT GTG GGT ACT AAT

96

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn			
20	25	30	
GTA GCC TGG TAT CAA CAG AAA CCA GGG CAA TCT CCT AAA GCA CTG ATT			144
Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile			
35	40	45	
TAC TCG GCA TCC TAC CGG TAC AGT GGA GTC CCT GAT CGC TTC ACA GGC			192
Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly			
50	55	60	
AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AAT GTG CAG TCT			240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser			
65	70	75	80
GAA GAC TTG GCA GAG TAT TTC TGT CAG CAA TAT AAC AGC TAT CCT CTC			288
Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Leu			
85	90	95	
ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGG GCT GAT GCT GCA			336
Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Ala			
100	105	110	

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9 VL region

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15
Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
20 25 30
Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
35 40 45
Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65 70 75 80
Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Leu
85 90 95
Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Ala
100 105 110

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9 heavy chain CDR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Tyr Trp Met His

1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None
(vii) IMMEDIATE SOURCE:
(B) CLONE: 3G9 heavy chain CDR2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys Asp Asn Glu Lys Phe Lys
1 5 10 15
Ser

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None
(vii) IMMEDIATE SOURCE:
(B) CLONE: 3G9 heavy chain CDR3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Thr Tyr Tyr Asp Ser Ser Phe Ala Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None
(vii) IMMEDIATE SOURCE:

(B) CLONE: 3G9 light chain CDR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(vii) IMMEDIATE SOURCE:

(B) CLONE: 3G9 light chain CDR2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Ala Ser Tyr Arg Tyr Ser
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(vii) IMMEDIATE SOURCE:

(B) CLONE: 3G9 light chain CDR3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln Gln Tyr Asn Ser Tyr Pro Leu Thr

1

5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 387 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZC 1-0

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence

- (B) LOCATION: 1...387

- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACC GGT GTC CAC TCC CAA GTC CAG CTT GTA CAG TCT GGG GCT GAG GTT	48
Thr Gly Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val	
1 5 10 15	
AAG AAG CCT GGG GCC TCA GTG AAG GTG TCC TGT AAG GCT TCT GGC AGT	96
Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Ser	
20 25 30	
ACC TTC ACC AGC TAC TGG ATG CAC TGG GTG AAG CAG GCG CCT GGA CAA	144
Thr Phe Thr Ser Tyr Trp Met His Trp Val Lys Gln Ala Pro Gly Gln	
35 40 45	
GGC CTT GAG TGG ATT GGA AGG ATT GAT CCA AAT AGT GGT GGT ACT AAG	192
Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys	
50 55 60	
GAT AAT GAG AAG TTC AAG AGC AAG GCC ACA CTG ACT GTA GAC AAA TCC	240
Asp Asn Glu Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Ser	
65 70 75 80	

ACC AGC ACA GCC TAC ATG GAG CTC AGC AGC CTG AGA TCT GAG GAC ACT	288
Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr	
85	90
	95
GCG GTC TAT TAT TGT GCA AGA GAG ACC TAC TAT GAT TCC TCG TTT GCT	336
Ala Val Tyr Tyr Cys Ala Arg Glu Thr Tyr Tyr Asp Ser Ser Phe Ala	
100	105
	110
TAC TGG GGC CAA GGG ACT ATG GTC ACT GTC TCT GCA GCT	375
Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ala Ala	
115	120
	125

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 129 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZHC 1-0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Thr Gly Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val			
1	5	10	15
Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Ser			
20	25	30	
Thr Phe Thr Ser Tyr Trp Met His Trp Val Lys Gln Ala Pro Gly Gln			
35	40	45	
Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys			
50	55	60	
Asp Asn Glu Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Ser			

65	70	75	80
Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr			
85	90	95	
Ala Val Tyr Tyr Cys Ala Arg Glu Thr Tyr Tyr Asp Ser Ser Phe Ala			
100	105	110	
Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ala Ala			
115	120	125	

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 387 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZHC S14G4PE

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...387
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACC GGT GTC CAC TCC CAA GTC CAG CTT GTA CAG TCT GGG GCT GAG GTT	48		
Thr Gly Val His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val			
1	5	10	15

AAG AAG TCT GGG GCC TCA GTG AAG GTG TCC TGT AAG GCT TCT GGC AGT	96	
Lys Lys Ser Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Ser		
20	25	30

ACC TTC ACC AGC TAC TGG ATG CAC TGG GTG AAG CAG GCG CCT GGA CAA	144	
Thr Phe Thr Ser Tyr Trp Met His Trp Val Lys Gln Ala Pro Gly Gln		
35	40	45

GGC CTT GAG TGG ATT GGA AGG ATT GAT CCA AAT AGT GGT GGT ACT AAG	192
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Gly	Leu	Glu	Trp	Ile	Gly	Arg	Ile	Asp	Pro	Asn	Ser	Gly	Gly	Thr	Lys	
50															60	
GAT AAT GAG AAG TTC AAG AGC AAG GCC ACA CTG ACT GTA GAC AAA TCC															240	
Asp	Asn	Glu	Lys	Phe	Lys	Ser	Lys	Ala	Thr	Leu	Thr	Val	Asp	Lys	Ser	
65															80	
ACC AGC ACA GCC TAC ATG GAG CTC AGC AGC CTG AGA TCT GAG GAC ACT															288	
Thr	Ser	Thr	Ala	Tyr	Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Thr	
85															95	
GCG GTC TAT TAT TGT GCA AGA GAG ACC TAC TAT GAT TCC TCG TTT GCT															336	
Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Glu	Thr	Tyr	Tyr	Asp	Ser	Ser	Phe	Ala	
100															110	
TAC TGG GGC CAA GGG ACT ATG GTC ACT GTC TCT GCA GCT															375	
Tyr	Trp	Gly	Gln	Gly	Thr	Met	Val	Thr	Val	Ser	Ala	Ala				
115															125	

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 129 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZHC S14G4PE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Thr	Gly	Val	His	Ser	Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val
1															15
Lys Lys Ser Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Ser															

20	25	30
Thr Phe Thr Ser Tyr Trp Met His Trp Val Lys Gln Ala Pro Gly Gln		
35	40	45
Gly Leu Glu Trp Ile Gly Arg Ile Asp Pro Asn Ser Gly Gly Thr Lys		
50	55	60
Asp Asn Glu Lys Phe Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Ser		
65	70	75
Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr		
85	90	95
Ala Val Tyr Tyr Cys Ala Arg Glu Thr Tyr Tyr Asp Ser Ser Phe Ala		
100	105	110
Tyr Trp Gly Gln Gly Thr Met Val Thr Val Ser Ala Ala		
115	120	125

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZLC 1-0

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence

- (B) LOCATION: 1...324

- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCT ACC GGT GTC CAC TCC GAT ATT GTC ATG ACT CAG TCT CCA TCA TCC	48		
Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser			
1	5	10	15

CTG TCC GCA TCA GTA GGA GAC AGG GTC ACC ATC ACC TGC AAA GCT TCT	96
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser	

20	25	30	
CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT CAA CAG AAA CCA GGG AAA Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys			144
35	40	45	
GCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAT CGG TAC AGT GGA GTC Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val			192
50	55	60	
CCT GAT CGC TTC TCA GGC AGT GGA TCC GGG ACA GAT TTC ACT CTC ACC Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr			240
65	70	75	80
ATC AGC AGT CTG CAG CCT GAA GAC TTC GCA ACG TAT TAC TGT CAG CAA Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln			288
85	90	95	
TAT AAC AGC TAT CCT CTC ACG TTC GGT GGT GGT ACC Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Thr			324
100	105		

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 108 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZLC 1-0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser

1	5	10	15
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser			
20	25	30	
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys			
35	40	45	
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val			
50	55	60	
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr			
65	70	75	80
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln			
85	90	95	
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Thr			
100	105		

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 3G9HZLC 1-0REI

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...324
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCT ACC GGT GTC CAC TCC GAT ATT GTC ATG ACT CAG TCT CCA TCA TCC	48
Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser	
1	5
10	15

CTG TCC GCA TCA GTA GGA GAC AGG GTC ACC ATC ACC TGC AAA GCT TCT	96
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser	
20	25
30	30

CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT CAA CAG AAA CCA GGG AAA	144		
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys			
35	40	45	
GCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAT CGG TAC AGT GGA GTC	192		
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val			
50	55	60	
CCT GAT CGC TTC TCA GGC AGT GGA TCC GGG ACA GAT TTC ACT TTC ACC	240		
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr			
65	70	75	80
ATC AGC AGT CTG CAG CCT GAA GAC ATC GCA ACG TAT TAC TGT CAG CAA	288		
Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln			
85	90	95	
TAT AAC AGC TAT CCT CTC ACG TTC GGT GGT GGT ACC	324		
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Thr			
100	105		

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 108 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

(B) CLONE: 3G9HZLC 1-0REI

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser			
1	5	10	15

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser
 20 25 30
 Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys
 35 40 45
 Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val
 50 55 60
 Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr
 65 70 75 80
 Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln
 85 90 95
 Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr
 100 105

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: HZLC 5-0

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...324
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCT ACC GGT GTC CAC TCC GAT ATT GTC ATG ACT CAG TCT CCA TCA TCC
 Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser
 1 5 10 15

48

CTG TCC GCA TCA GTA GGA GAC AGG GTC ACC ATC ACC TGC AAA GCT TCT
 Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser
 20 25 30

96

CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT CAA CAG AAA CCA GGG AAA	144		
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys			
35	40	45	
GCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAT CGG TAC AGT GGA GTC	192		
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val			
50	55	60	
CCT GAT CGC TTC TCA GGC AGT GGA TCC GGG ACA GAT TTC ACT TTC ACC	240		
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr			
65	70	75	80
ATC AGC AGT CTG CAG CCT GAA GAC TTC GCA ACG TAT TAC TGT CAG CAA	288		
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln			
85	90	95	
TAT AAC AGC TAT CCT CTC ACG TTC GGT GGT GGT ACC	324		
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr			
100	105		

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 108 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: HZLC 5-0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser

1 5 10 15

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser

20	25	30
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys		
35	40	45
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val		
50	55	60
Pro Asp Arg Phe Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr		
65	70	75
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln		
85	90	95
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr		
100	105	

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA.

(vii) IMMEDIATE SOURCE:

- (B) CLONE: HZLC 6-0

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence

- (B) LOCATION: 1...324

- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCT ACC GGT GTC CAC TCC GAT ATT GTC ATG ACT CAG TCT CCA TCA TCC				48
Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser				
1	5	10	15	
CTG TCC GCA TCA GTA GGA GAC AGG GTC ACC ATC ACC TGC AAA GCT TCT				96
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser				
20	25	30		

CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT CAA CAG AAA CCA GGG AAA	144		
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys			
35	40	45	
GCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAT CGG TAC AGT GGA GTC	192		
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val			
50	55	60	
CCT GAT CGC TTC TCA GGC AGT GGA TCC GGG ACA GAT TTC ACT CTC ACC	240		
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr			
65	70	75	80
ATC AGC AGT CTG CAG CCT GAA GAC ATC GCA ACG TAT TAC TGT CAG CAA	288		
Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln			
85	90	95	
TAT AAC AGC TAT CCT CTC ACG TTC GGT GGT GGT ACC	324		
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Thr			
100	105		

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 108 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (B) CLONE: HZLC 6-0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ala Thr Gly Val His Ser Asp Ile Val Met Thr Gln Ser Pro Ser Ser			
1	5	10	15
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser			

20	25	30
Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys		
35	40	45
Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val		
50	55	60
Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr		
65	70	75
Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln		
85	90	95
Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr		
100	105	

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

- (B) CLONE: REI-con

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly		
1	5	10
Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr		
20	25	30
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile		
35	40	45
Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly		
50	55	60
Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro		
65	70	75
Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln		
85	90	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/10284

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 16/18, 16/28; A61K 39/395; C12N 15/12, 15/13, 15/14

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8, 12, 885; 530/387.1, 387.3, 388.1, 388.22; 424/139.1, 143.1, 144.1; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,843,726 A (LEE) 01 December 1998 (01.12.98), see entire document, especially column 3, lines 10-15.	1-5, 39

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"R"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 JULY 2000

Date of mailing of the international search report

10 AUG 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230Authorized officer
PREMA MERTZ
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/10284

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

514/2, 8, 12, 885; 530/387.1, 387.3, 388.1, 388.22; 424/139.1, 143.1, 144.1; 536/23.1, 23.5

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, CAS ONLINE, MEDLINE, CAPLUS

search terms: erythropoietin receptor antibody, method, treatment, administer, therapy, erythropoiesis, anemia, cytopenia, nucleic acid, chimeric antibody

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/10284

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority; namely:

2. Claims Nos.: 6-38 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 6-38 recite a specific SEQ ID NO encompassing a specific amino acid sequence which cannot be searched because the computer readable form submitted has been found to be defective.

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II. Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
..
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
..

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.